STUDIES OF THE STRUCTURE OF THE ACTIVE SITE OF

DIHYDROPTERIDINE REDUCTASE FROM

HUMAN BRAIN

A thesis submitted for

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by

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CERTIFICATE OF ORIGINALITY

The work described in this thesis was carried out by the candidate at The Australian National University, unless otherwise stated.

Akiko Ohnishi

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A.O.

Abbreviations

alk.	alkaline
analyt.	analytical
app.	apparent
approx.	approximate
BSA	bovine serum albumin
ca	circa (= about)
cf.	confer (= compare)
concn	concentration
dec.	decomposed
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
I_{50}	concentration of inhibitor causing 50% inhibition
k_{cat}	the turnover number
Kd	dissociation constant
Ki	inhibition constant
Km	Michaelis constant
max	maximum
6-MeDHP	6-methyl-7,8-dihydro(6H)pterin
6-MeTHP	6-methyl-5,6,7,8-tetrahydropterin
6,7-Me_2THP	6,7-dimethyl-5,6,7,8-tetrahydropterin
Mr	relative molecular weight
Mol. Wt.	molecular weight
NEM	N-ethylmaleimide
neut	neutral
O.D.	optical density, absorbance
PAGE	polyacrylamide gel electrophoresis

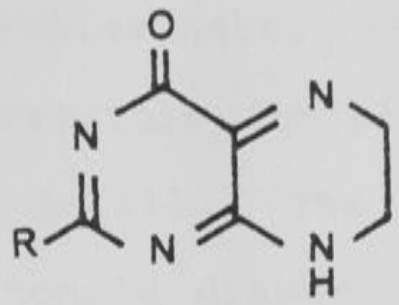
PCMB	p-chloromercuribenzoic acid
pterin	2-aminopteridin-4(3H)-one
-SH	thiol group
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
UV	ultraviolet
V_{max}	maximum velocity

ABSTRACT

The aim of this work was to gain information about the nature of the active site of human brain dihydropteridine reductase. This included the determination of i) the amino acid composition, ii) the stoichiometry of the nucleotide cofactor and the enzyme, iii) the predominant tautomeric structure of the active substrate, iv) the effect of platinum(II) complexes on enzyme activity, and v) the nature of thiol groups of the protein.

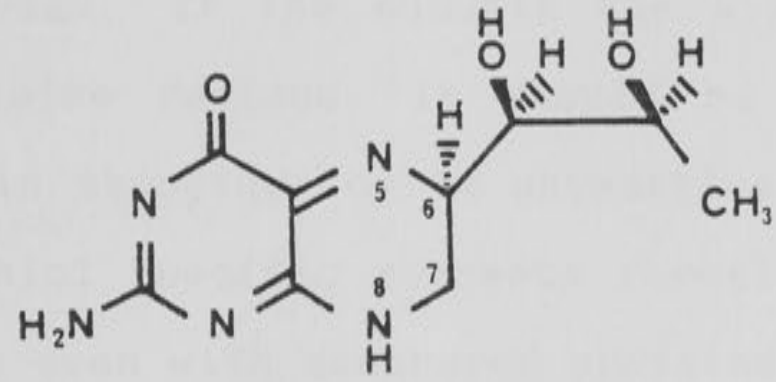
Amino acid analysis revealed that the amino acid composition of human brain DHPR was the same as the human liver enzyme within experimental error. The protein concentration which was obtained from the amino acid analysis gave the same value as the one from the Bio-Rad microassay, whereas the Lowry method gave almost twice the value of the above two methods for human brain DHPR. So in this thesis the protein concentration was measured by the Bio-Rad microassay. By using this method, the extinction coefficient of this protein was found to be 92,800 $M^{-1}.cm^{-1}$ at 280nm. The stoichiometry of NADH to enzyme (from various mammalian sources) has been reported during the last few years by many laboratories. This work revealed that the ratio of NADH to the enzyme from human brain was 1 : 1.

The predominant enzyme active tautomer of the substrate was shown to have the ortho quinonoid structure [1] in this work, because the 2-unsubstituted pteridin-4(3H)-one derivatives, 2-methyl, 2-methylthio and 2-methyl-



[1]

amino derivatives were good substrates of human brain DHPR, while tetrahydrolumazine or 2-thiolumazine derivatives were not. This also indicates that DHPR can tolerate various substituents in the pteridine ring, and that the 2-amino group of the natural substrate, quinonoid dihydrobiopterin [2], is not totally essential for substrate activity. It



[2]

may, however, have an important role in binding to the active site of the enzyme. Although attempts were made, it was not possible to obtain information about the charges at the amino acid residues which were essential for enzyme catalysis because of the instability of the quinonoid species and NADH cofactor at non-neutral pH.

A brief study of the inhibition of DHPR by platinum complexes was made, but none of the compounds, e.g. H_2PtCl_6 [I_{50} 360 μM (4 h incubation)], trans-platin [I_{50} 1 mM (15 min)], were particularly strong inhibitors.

Potassium tetrachloroplatinate, on the other hand, was shown to be an irreversible inhibitor. This inactivation was studied in some detail. The enzyme was protected by NADH and by the quinonoid dihydro(6H)pterin cofactor from this inactivation.

The nature of the thiol groups of this enzyme were revealed by using thiol specific reagents and potassium tetrachloroplatinate. Each DHPR subunit (the enzyme has two identical subunits) has 3 or 4 cysteine residues. One of them is located at the outer surface of the subunit and two of them seem to be located at or near the active site of the enzyme. If the subunit has a further (i.e. the fourth) cysteine residue, it should be located deep inside the protein structure or is unreactive, because the reactions with thiol specific reagents revealed only three cysteine residues even with denatured proteins. Amino acid analysis showed that four residues may possibly be present.

TABLE OF CONTENTS

CHAPTER 2 AMINO ACID COMPOSITION, PROTEIN CONCENTRATION, AND SPECTROSCOPIC PROPERTIES AND REACTIVITY OF THIOL GROUPS

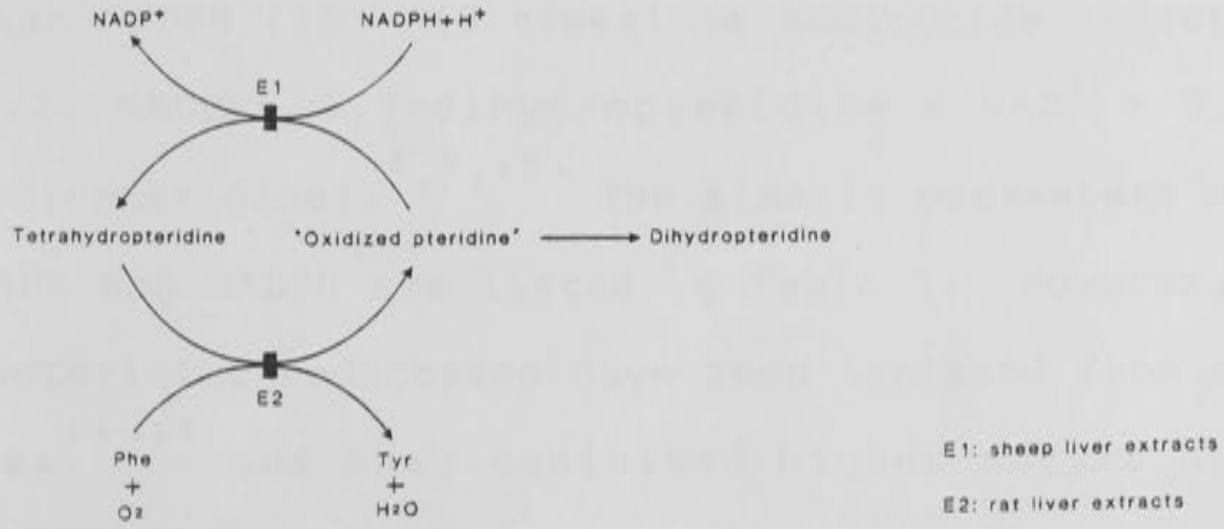
CHAPTER 3 NEW PTERIDINE SUBSTRATES FOR DHPR

CHAPTER 1

GENERAL INTRODUCTION

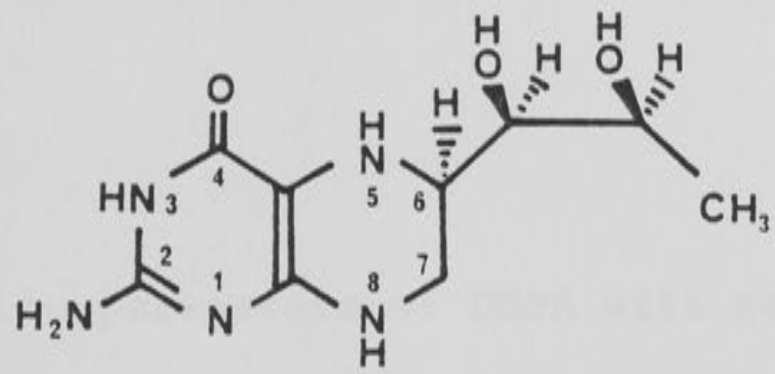
1-1 History of the discovery of Dihydropteridine reductase

The conversion of phenylalanine into tyrosine was especially interesting because this oxidation was at least partially blocked in the disease, oligophrenia phenylpyruvica,[1] and had been demonstrated in cell free extracts in 1913.[2] In 1952, Udenfriend and Cooper reported that rat liver extracts had the ability to catalyze this reaction and that NAD^+ and an alcohol or aldehyde were required for enzyme activity.[3] Kaufman found that this system required two enzymes (i.e. a rat liver and a sheep liver enzyme) and that NADPH and oxygen were also necessary.[4] He found later that the rat liver enzyme was phenylalanine hydroxylase which catalyzed the oxidation of phenylalanine to tyrosine and the sheep liver enzyme catalyzed the reaction involving NADPH which served to maintain the cofactor in the active, reduced form (Scheme 1).[5,6]



Scheme 1

At that stage, the structure of the pteridine cofactor for the rat liver enzyme was not yet known, but in 1963 Kaufman found that the cofactor was L-erythro-5,6,7,8-tetrahydrobiopterin [1].[7] In his preparations, the rat



[1]

liver enzyme was freshly extracted but the sheep liver enzyme was kept at -20^{0}C after death and thawed before extraction. Phenylalanine hydroxylase activity decreased rapidly after death but loss of activity on storage was slower at -20^{0}C. In 1972, Craine et al. found that the NADPH dependency for the reductase was due to dihydrofolate reductase which contaminated the liver enzyme preparation.[8] Later the sheep liver reductase, now called dihydropteridine reductase (DHPR) was found to be more active with NADH than NADPH (15 ~ 45 times) as nucleotide cofactor (EC 1.6.99.7, NADH + 6,7-dihydropteridine = NAD^+ + 5,6,7,8-tetrahydropteridine).[8,9,10] The kinetic parameters of DHPR with NADH and NADPH are listed in Table 1. However, other dihydropteridine reductases have been isolated from several sources,[11~14] and they exhibited higher enzyme activity with NADPH than with NADH as cofactor. The same Enzyme Commission number, E.C. (1.6.99.7.) was given for these enzymes.

Table 1. Kinetic parameters of DHPR with NADH and NADPH.

Enzyme	NADH			NADPH			ref.
	app.K_m [1]	app.V_{max} [2]	S	app.K_m [1]	app.V_{max} [2]	S	
h.b.	4.7	336	90	——	——		15
h.l.	8.0	1.8	12	110	0.59	12	8
h.l.	29	468	75	770	316	75	10
h.p.	12	0.09	10	——	——		16
s.b.	20	——		——	——		17
s.l.	30	92	75	300	2.3	75	17
b.l.	9.1	167	?	——	——		18
r.l.	2.2	2.45	12	100	0.88	12	8

[1] µM; [2] µmol NAD(P)H oxidized/min per mg of protein;

S concentration of quinonoid pteridine used, µM.

h.b. human brain; h.l. human liver; h.p. human platelets; s.b. sheep brain; s.l. sheep liver; b.l. bovine liver; r.l. rat liver.

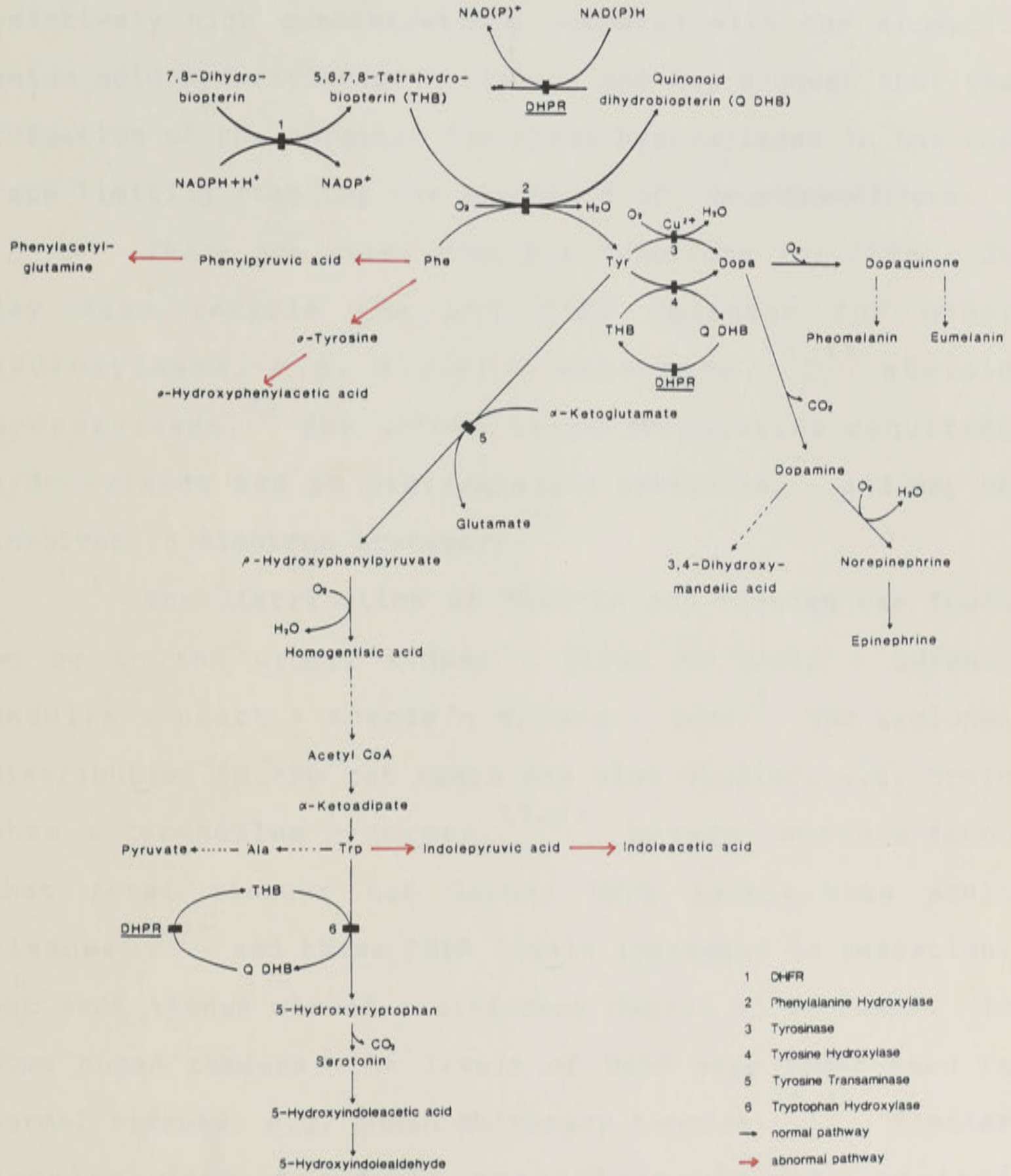
DHPR usefully recycles the pteridine cofactor for aromatic amino acid hydroxylases, i.e. phenylalanine hydroxylase[19] (phenylalanine to tyrosine), tyrosine hydroxylase[20] (tyrosine to dopa), and tryptophan hydroxylase[21] (tryptophan to 5-hydroxytryptophan).

1-2 Occurrence and distribution

Dihydropteridine reductase is widely distributed in mammalian tissues in which it plays an important role in metabolism (Table 2, cf. reference 22). The aromatic amino acid hydroxylases with which DHPR is associated are of importance especially for the synthesis of neurotransmitters and lack of these enzymes or of the pteridine cofactor can cause serious neurological problems. The relationship between the aromatic amino acid hydroxylases, their cofactor, substrates and some metabolites are shown in Scheme 2.[23] Phenylalanine is an essential amino acid for protein synthesis in mammalian tissues. Physiologically, its most significant degradation pathway involves its hydroxylation to tyrosine. This hydroxylation is restricted mainly to the liver, kidney and pancreas in mammals. Tyrosine, the product of hydroxylation is also an essential amino acid for protein synthesis, and in the central nervous system it is the precursor of the neurotransmitters, dopamine, norepinephrine and epinephrine. Tryptophan is also one of the essential amino acid for mammals and is the precursor of another neurotransmitter,

Table 2. Sources from which DHPR has been isolated (*cf.* reference 22).

Human	brain, liver, lung, breast (normal and neoplastic cells), fetal tissues, placenta, amniotic cells, peripheral blood, platelets, lymphocytes, continuous lymphoid cells, skin fibroblasts
Monkey	brain, liver
Bovine	brain, pineal gland, liver, kidney, adrenal medulla
Sheep	brain, liver, adrenal medulla
Rabbit	liver
Cat	liver
Rat	brain, liver, heart, lung, kidney, adrenal medulla, spleen, muscle, uremic tissues, erythrocytes, pheochromocytoma
Mouse	RAG cells, C-6 Glioblastoma, C-1300 Neuroblastoma
Hamster	Chinese WOR cells, Syrian TG2 cells
Fish	*Cheirodon axelrodi*, *Salmo irideus*
Protozoan	*Tetrahymena*, *Crithidia fasciculata*
Bacteria	*Pseudomonas* species (ATCC 11299a)


NAD(P)+
NAD(P)H
DHPR
7,8-Dihydro-biopterin
5,6,7,8-Tetrahydro-biopterin (THB)
Quinonoid dihydrobiopterin (Q DHB)
1
NADPH+H+
NADP+
2
O2
H2O
Cu2+
3
4
Phenylacetyl-glutamine
Phenylpyruvic acid
Phe
Tyr
Dopa
Dopaquinone
Pheomelanin
Eumelanin
THB
Q DHB
DHPR
p-Tyrosine
p-Hydroxyphenylacetic acid
α-Ketoglutamate
5
CO2
Dopamine
Glutamate
p-Hydroxyphenylpyruvate
3,4-Dihydroxy-mandelic acid
Norepinephrine
Epinephrine
Homogentisic acid
Acetyl CoA
α-Ketoadipate
Pyruvate
Ala
Trp
Indolepyruvic acid
Indoleacetic acid
6
5-Hydroxytryptophan
Serotonin
5-Hydroxyindoleacetic acid
5-Hydroxyindolealdehyde
1 DHFR
2 Phenylalanine Hydroxylase
3 Tyrosinase
4 Tyrosine Hydroxylase
5 Tyrosine Transaminase
6 Tryptophan Hydroxylase
normal pathway
abnormal pathway

Scheme 2

serotonin.

Craine et al.[8] reported that DHPR was in relatively high concentrations compared with the aromatic amino acid hydroxylases in tissues and may suggest that the formation of the cofactor for these hydroxylases is not the rate limiting step for the synthesis of neurotransmitters.

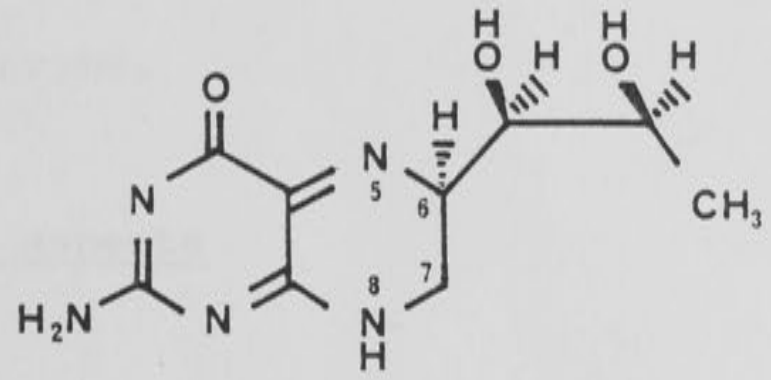
There are other possible functions for DHPR. It may also recycle the pteridine cofactor for other hydroxylases, e.g. glycerol etherases,[24,25] steroid hydroxylases,[26] yet unkown tetrahydropterins requiring hydroxylases and in prostaglandin synthesis,[27] and may be involved in electron transport.[28]

The distribution of DHPR in rat tissues was found to be in the order, kidney > liver >> brain > adrenal medulla > heart > muscle = spleen > lung.[8] The regional distribution in the rat brain was also studied, e.g. brain stem > cerebellum > cortex.[29~31] Several workers found that fetal tissues had lesser DHPR levels than adult tissues[32~34] and these DHPR levels increased in gestation, but each tissue showed a different degree of increase. In some human tumours, the levels of DHPR were lower than in normal tissues, e.g. human pulmonary tumours.[32,34] Similar results were found in transplantable rat ascites hepatoma.[35] On the other hand, in the case of breast cancer,[36] and neoplastic clonic tissue,[37] the DHPR levels were higher than in normal cells. Cultures from C-6 mouse glioblastoma also possessed high DHPR activity compared with those from C-1300 mouse neuroblastoma which had

negligible activity.[31] DHPR levels in human peripheral leucocytes, erythrocytes, lymphocytes and granulocytes are good indicators for diagnosis purposes, i.e. a rapid method for checking malignancy.[38~41] The enzyme was also isolated from human platelets[17,42] and the activity was found to decrease with aging.[42] Human continuous lymphoid and amniotic cells also contain DHPR and they have been used for the purpose of early diagnosis of variant forms of hyperphenylalaninemia[41,43,44] (cf. Section 1-3, p 10).

Other sources of DHPR are shown in Table 2. Some mutants of Tetrahymena are DHPR or phenylalanine hydroxylase deficient and can be used as models for conditions similar to Phenylketonuria in humans.[45] DHPR was also isolated from Crithidia fasciculata which had some form of biopterin reduction system in the cell.[46]

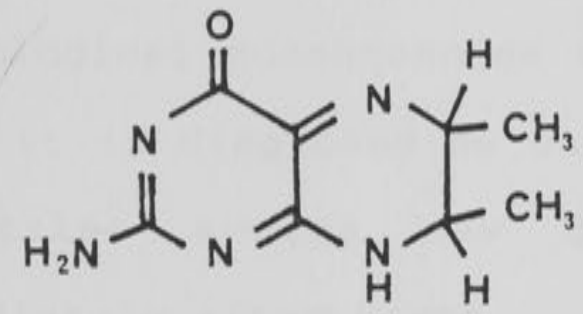
Among these sources of DHPR (Table 2), quinonoid L-erythro-7,8-dihydro(6H)biopterin [2] was the natural



[2]

substrate and was found to be a superior substrate than other quinonoid pteridines which are derived from synthetic pteridines (e.g. 6,7-dimethyl-7,8-dihydro(6H)pterin

[3]).[8,10,47~49] This was not the case in Pseudomonas species (ATCC 11299a), which was found to have no biopterin or its reduced forms in the cells. Quinonoid L-threo-7,8-dihydro(6H)neopterin [4] was found to be a superior



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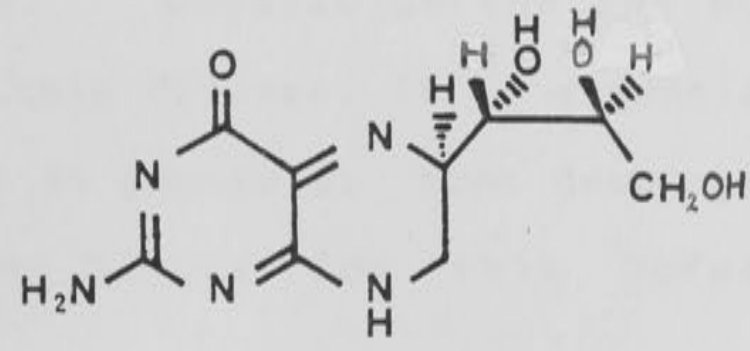


[4]

cofactor for the enzyme and L-threo-neopterin was present in the cells of this bacterium.[50] The Pseudomonas DHPR required NADH as the nucleotide cofactor and was inactive when the cofactor was replaced by NADPH.[51]

DHPR was also detected in the fish species Cheirodon axelrodi and Salmo irideus.[52]

The reductase has always been isolated from the soluble fractions of tissues, i.e. DHPR is a soluble cytoplasmic enzyme.

1-3 Clinical aspects

1-3-1 Phenylketonuria and Hyperphenylalaninemia

Phenylketonuria (PKU) and Hyperphenylalaninemia belong to a group of inborn errors of metabolism of phenylalanine which shows impaired phenylalanine oxidation, and results in elevated tissue and serum phenylalanine.[53]

The classification of Hyperphenylalaninemia and related defects is summarized in Table 3 (cf. reference 53). PKU affects 1 in ca 22,000 births,[54] but can be as high as 1 in 10,000[55] in certain populations. PKU is an autosomal recessive inherited disease.[56] Because of the serious neurological consequences of this disease, it is essential that it is diagnosed as early as possible. Most developed countries screen new born babies for this defect immediately after birth.

The biochemical aspects of PKU are now well characterized. In the more common classical form (type I and II in Table 3) the patients have low or no phenylalanine hydroxylase in their tissues. They are unable to convert phenylalanine to tyrosine and other metabolic pathways became activated (cf. Scheme 2). The neurological problems are usually corrected by using low phenylalanine diets.

1-3-2 Hyperphenylalaninemia due to DHPR deficiency

DHPR deficiency (type IV in Table 3) was confirmed by Kaufman et al. in 1975.[57] The patients had no DHPR in the liver, brain and skin fibroblasts. The phenylalanine hydroxylase activity was 20% of the average normal adult value and the urine and blood contained almost no reduced biopterin with very high xanthopterin and dihydrobiopterin, as well as traces of biopterin and neopterin.[58] This deficiency causes a decrease in the amounts of neurotrans-

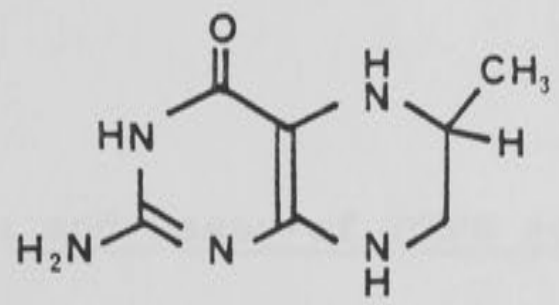
Table 3. Classification of Hyperphenylalaninemia.

Type	Condition	Defect	Clinical aspects
I	Phenylketonuria	Phenylalanine hydroxylase absent	Mental retardation and associated symptoms if untreated
II	Persistent hyper-phenylalaninemia	Decreased Phenylalanine hydroxylase	Normal; may show retardation if untreated in more severe cases
III	Transient mild hyperphenyl-alaninemia	Maturational delay of Phenylalanine hydroxylase	Normal
IV	DHPR deficiency	DHPR deficient or absent	Initially normal; seizures, abnormal development evident within first year of life; death before age of 10 years
V	Abnormal dihydrobiopterin function	Dihydrobiopterin synthesis defect	Myoclonus, uncontrolled movements, tetraplegia, greasy skin, recurrent hyperthermia

mitter precursors in the brain because the biopterin cofactor for the hydroxylases cannot be recycled. Also the levels of the metabolites of dopamine and serotonin, i.e. homovanillic acid and 5-hydroxyindoleacetic acid are decreased in the brain and the cerebrospinal fluid.

This deficiency can be detected by assaying for DHPR activity in liver biopsies,[57,59] skin fibroblast cultures[44,60] and/or blood cells,[38,39,44] or by using antigen-antibody reactions (e.g. reference 41).

The clinical treatment involves administration of L-dopa, 5-hydroxytryptophan and carbidopa, and sustaining a low phenylalanine diet. Tetrahydrobiopterin has also been used as a drug in these cases. Moreover, the analogue of tetrahydrobiopterin, 6-methyl-5,6,7,8-tetrahydropterin [5] has been used on occasions, but apparently unlike the natural cofactor, it is hepato toxic.[61]



[5]

1-3-3 Genetic aspects

PKU is rare among Negroes and Orientals but is common in Caucasians. It is more prevalent among Celtic people and Central Europeans.[62]

Firgaira et al.[41] found more than one DHPR protein in the same patients and suggested that one DHPR protein species was derived from the other, implying that the same structural gene(s) encodes for DHPR in human liver, lymphocytes, and fibroblasts. They also demonstrated the possibility of DHPR gene mutations by comparing the immunoreactivity of cultured cell extracts from DHPR deficient children and their parents. It should be noted that recently Webber et al.[63] haye shown three NADH-dependent DHPR species from rat liver by isoelectric focusing and demonstrated that the protein species differed probably by minor structure changes, e.g. glutamic acid to glutamine, which occurred in the cell, because their N-terminal and amino acid analyses showed no significant differences.

1-4 Purification and assay of DHPR activity

1-4-1 Purification

DHPR has been purified by using classical techniques (e.g. protein precipitation, adsorption and ion-exchange chromatography); and affinity chromatography. In most procedures, the enzyme was precipitated from the crude

extract by ammonium sulphate at 40 ~ 70% salt saturation. When Craine et al.[8] purified DHPR from sheep liver, they used several steps to obtain pure enzyme, i.e. ammonium sulphate precipitation, zinc ethanol fractionation, alkaline ammonium sulphate precipitation, elution from calcium phosphate gel, DEAE-cellulose chromatography, and Sephadex G-100 gel filtration (9% yield and 160-fold purification). In most cases now affinity column chromatography followed by ion-exchange chromatography is used routinely directly after ammonium sulphate fractionation.

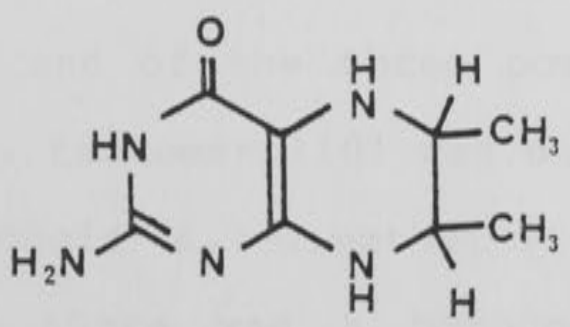
Human liver DHPR was purified by Firgaira et al.[10] by using a naphthoquinone-AH-Sepharose affinity column (26% yield, 1000-fold purification). Shen et al.[42] purified human platelet DHPR using a Matrex Gel Blue column as an affinity adsorbant (46% yield, 1500-fold purification). As above, affinity column chromatography shortened the purification procedure because of the greater specificity of the column material, although in all the cases at least three steps were still required. This is because the specificity is far from complete. Nakanishi et al.[64] reported the purification of human liver DHPR using only a two step procedure, i.e. passage of the extract through a Matrex Gel Blue column followed by a phenyl Sepharose column (30% yield and 700-fold purification).

Several different affinity columns were used by different laboratories (cf. reference 22). Some columns function by binding to the nucleotide binding site of the

enzyme, e.g. Matrex Gel Blue, 5'-AMP Sepharose, and others to the pteridine binding site, e.g. naphthoquinone, phenyl Sepharose, of the enzyme. The human brain enzyme does not bind to the naphthoquinone column unless the solutions contain NADH and the column is in the oxidized form. The enzyme is released from this column by buffer free from NADH and containing a reducing agent, dithiothreitol (DTT).

1-4-2 Substrate specificity

Kaufman[65] reported that the second natural cofactor in addition to NADPH for sheep liver DHPR (see Scheme 1, p 1) was a pteridine derivative. It first had to be reduced by this enzyme with a pyridine nucleotide cofactor before it was an effective cofactor for the hydroxylases (e.g. phenylalanine hydroxylase). In a detailed study he showed that a synthetic pterin, 6,7-dimethyl-5,6,7,8-tetrahydropterin [6] could replace the boiled liver extract as cofactor and using synthetic models he showed that the pterin cofactor was a double bond tautomer of 7,8-dihydropterin, probably the 5,6-dihydro isomer [7].[66] The natural cofactor for DHPR was isolated



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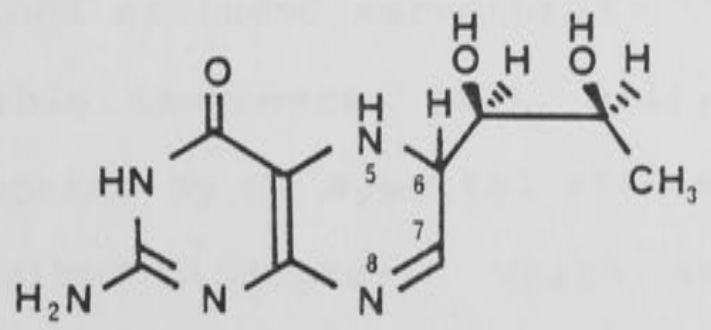
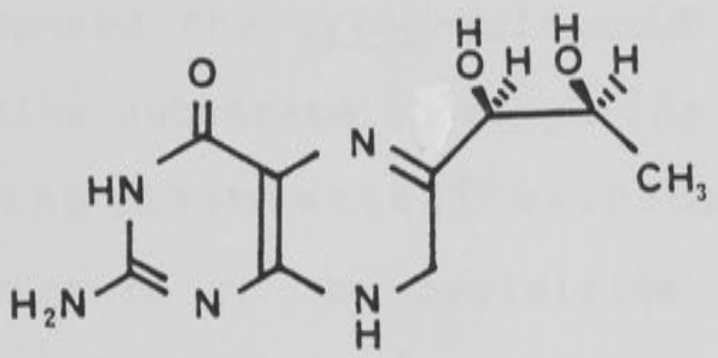


[7]

by Kaufman in an oxidized form and shown to be L-erythro-7,8-dihydro(3H)-biopterin [8]. This was a substrate for dihydrofolate reductase which reduced it to the tetrahydro derivative using NADPH as a cofactor.[67] Further identity of the cofactor was obtained by oxidation to biopterin and characterized by the Crithidia bioassay which was very sensitive for the L-erythro isomer of biopterin. He suggested, however, later that there were several



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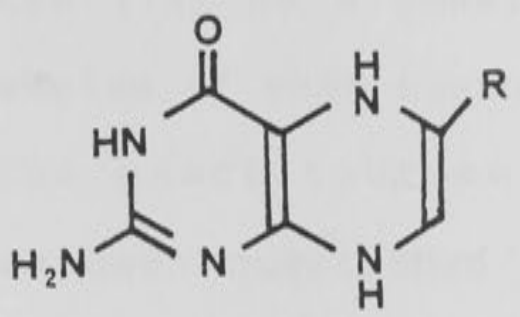
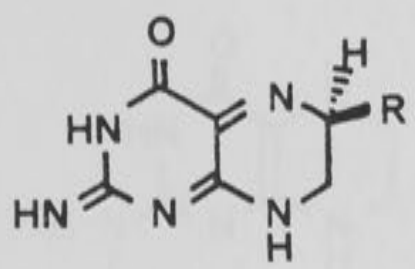
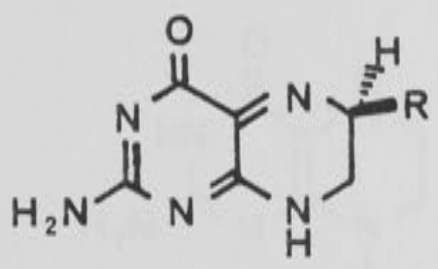


[9]

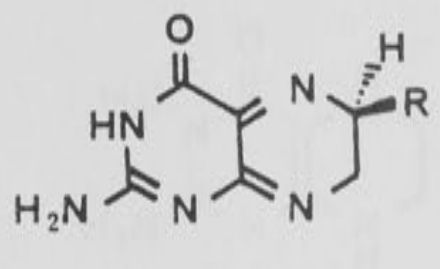
tautomeric possibilities, e.g. [9], [10], [11], and [12], in addition to structure [7] for the active pterin cofactor.



[10]



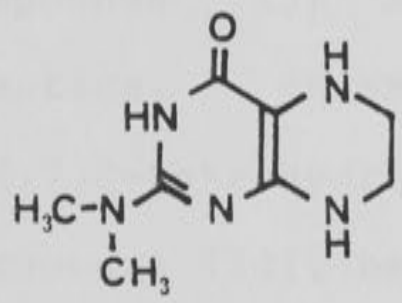
[11]



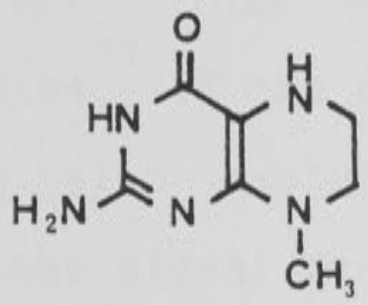
[12]

Several investigations excluded structures [7] and [9], and of the three possible tautomers, [10], [11], and [12], tautomer [10] was supported by UV spectral studies of quinonoid 6,7-dimethyl-7,8-dihydro(6H)pterin which showed that there was a bathochromic shift of 15 ~ 20 nm in

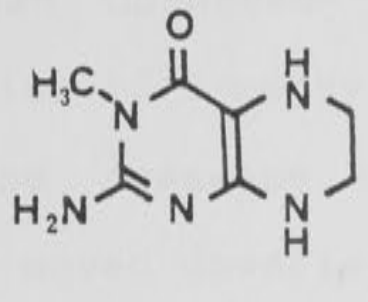
the 2-alkylamino derivatives whereas no such shift was observed in 8-alkylated derivatives.[68] But in this study 3-alkylated derivatives were not examined, so that structure [11] was not strictly excluded. Viscontini and Bobst[69] suggested that structure [10] was a possible tautomer by comparing the oxidation of several N-methyl-tetrahydro derivatives. Also Bobst supported structure [10] by molecular orbital calculation.[70] Gready later proposed the ortho-quinonoid structure [12] as a possible active substrate by comparing the energies of each tautomer using theoretical methods.[71] The exact tautomeric structure of the pyrimidine ring has been questioned and kinetic evidence was presented by Armarego and Waring[72] which showed that tautomer [11] was the reactive tautomer. They compared the rates of aerobic oxidation of 2-dimethylamino- [13], 8-methyl- [14], and 3-methyl-5,6,7,8-tetrahydropterin [15] derivatives and found that the rates



[13]



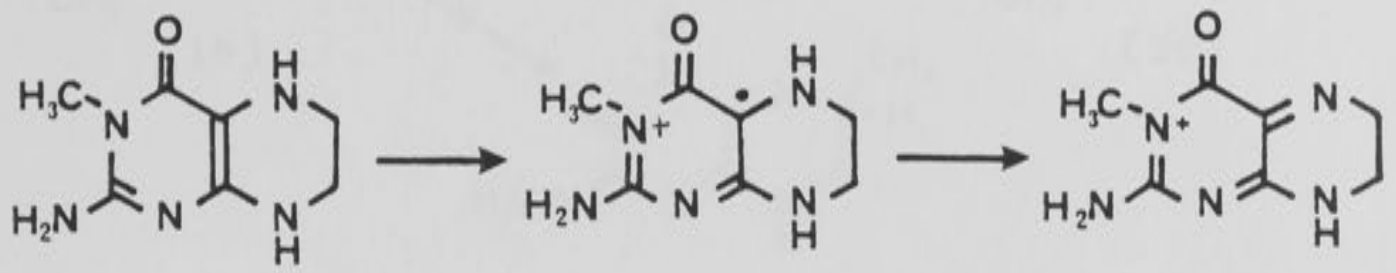
[14]



[15]

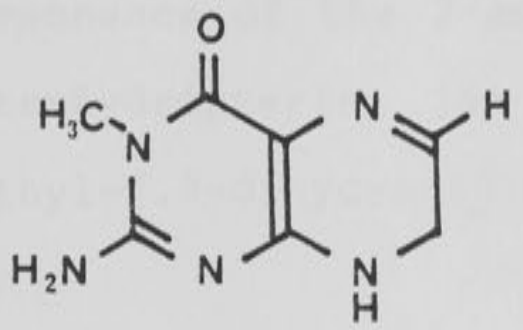
of oxidation of compounds [13] and [14] were faster than compound [15]. This was explained by stabilization of the initial radical cation intermediate [16] leading to the cation [17]. Such stabilization does not occur in the 3-unsubstituted pterins, [13] and [14], because the radical

cation would be deprotonated at the neutral pH of the oxidation buffer by loss of H^+ from N-3. In the 3-methyl



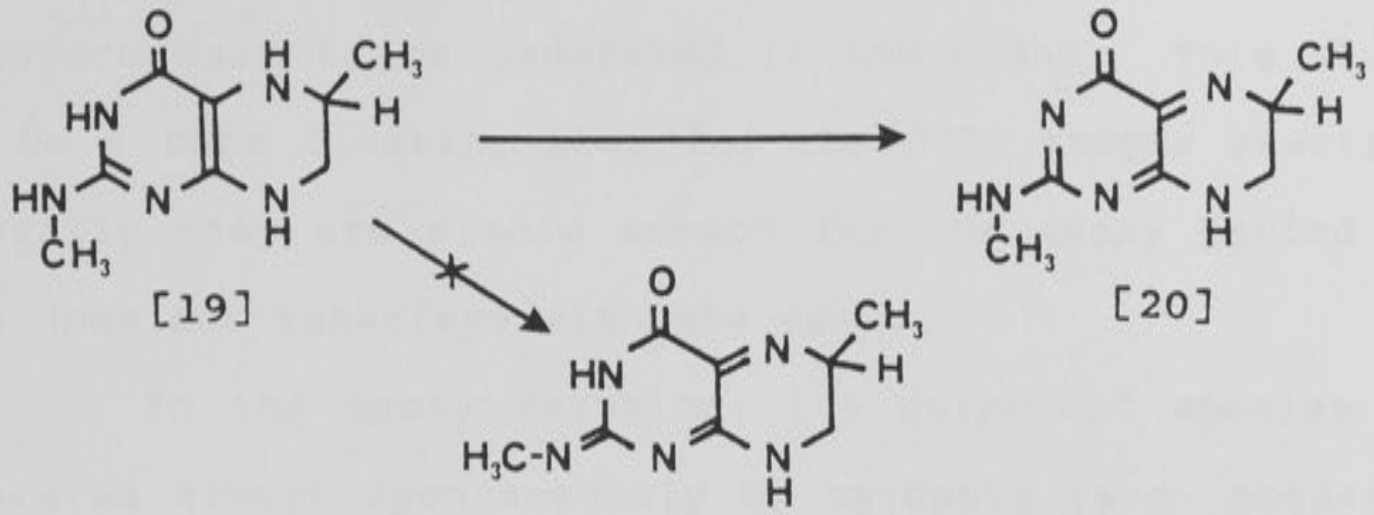
[15] [16] [17]

compound [15], a positive charge will be generated at N-3 to give the quinonoid pterin [17] which should rearrange to the corresponding 7,8-dihydro(3H)pterin [18] much faster than the corresponding quinonoid pterins derived from



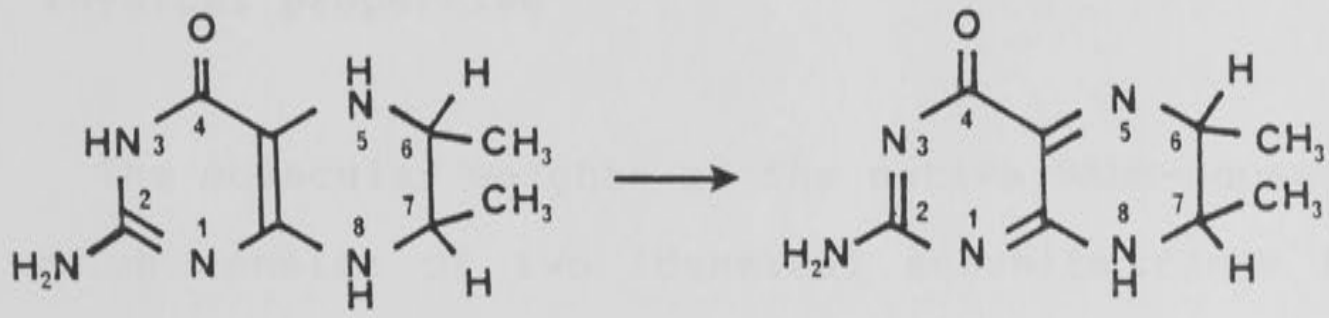
[18]

compounds [13] and [14]. This has been observed in practice. ^{1}H NMR studies[73] of the oxidation of 6-methyl-5,6,7,8-tetrahydropterin also excluded the presence of structure [12], because the signal from H-6 moved downfield by 1.08 ppm whereas the signals from H-7 axial and equatorial were shifted downfield by only 0.27 and 0.18 ppm, respectively. Moreover, oxidation of 6-methyl-2-methylamino-5,6,7,8-tetrahydropterin [19] did not show large chemical shift changes of the 2-methylamino protons which implied that structure [20] was formed. This result could not be extrapolated to the 2-unsubstituted pterins



[19] [20] [21]

because methylation of the C-2 amino group might perturb the tautomeric equlibrium to form the exocyclic tautomer [21]. Benkovic et al.[74] later used ^{15}N NMR spectroscopy to demonstrate clearly that tautomer [11] was the major species in the buffer solution. The downfield chemical shift of the ^{15}N resonance of the 2-amino group when 6,7-dimethyl-5,6,7,8-tetrahydropterin [6] was oxidized to quinonoid 6,7-dimethyl-7,8-dihydro(6H)pterin [3] is too



[6] [3]

small (~41 ppm) to be consistent with an $sp^3 \rightarrow sp^2$ change (i.e. $C-NH_2 \rightarrow C=NH$); compare the chemical shift of N-5 upon oxidation which undergoes a 320 ppm downfield shift.

1-4-3 Assay methods

The true pteridine subsrates for DHPR are the quinonoid species. These are relatively unstable and

therefore have to be generated in the assay. This should not be a rate limiting step for the DHPR enzyme reaction. Generally they are stable enough for the assay period and this does not interfere with the assay.

In the assay solution, the quinonoid species are generated almost spontaneously by oxidants (e.g. potassium ferricyanide, hydrogen peroxide or peroxidase in the presence of hydrogen peroxide). The solution, in general, contains an oxidant, tetrahydropteridine, NADH, buffer and the reaction is initiated by the additon of DHPR. The rate of the reduction of quinonoid pteridine to tetrahydropteridine is observed by the absorbance changes at 340 nm due to the concurrent oxidation of NADH to NAD^+ using double beam spectrometer.

1-4-4 Physical properties

The molecular weights of the native NADH-dependent DHPRs which consist of two identical subunits range from 41,000 to 55,000 and vary depending on the enzyme sources and the methods of estimation. For example, on PAGE, human liver DHPR showed very high Mr values (e.g. 100,000) because of its relatively low charge to size ratio.[10] On gel chromatography, on the other hand, it showed an Mr value of 47,500, and sedimentation-equilibrium analysis gave a value of 50,000.

The molecular weight of the subunit is between 25,000 ~ 27,000. For example, in the above human liver

Table 4. Physical properties of Dihydropteridine Reductase

Enzyme	Isoelectric point	Optimum* pH	Optimum* temperature (^0C)	ε_{280} ($M^{-1}.cm^{-1}$)	ref.
h.l.	7.0	7.2	37		10
b.l.	5.7			90.000	75
				83,000	76
b.a.m.	5.7				75
b.b.	5.7				77
s.l.	5.4	6.8~7.0			78 17,78
				88,500	78
r.l.	6.4	6.3~6.8		105,000	78
	5.7, 5.9 and 6.5				63
P.		7.2			51

* for enzyme activity

h.l. human liver; b.l. beef liver; b.a.m. beef adrenal medulla; b.b. beef brain; s.l. sheep liver; r.l. rat liver; P. Pseudomonas.

DHPR, SDS-PAGE revealed a subunit Mr value of 26,000.[10]

The measured isoelectric points are listed in Table 4. Among these, human liver DHPR has a relatively high (7.0)[10] isoelectric point compared with others (5.4 ~ 6.4) (cf. reference 22). The optimum pH and temperature for DHPR activity and also the extinction coefficients for DHPR are listed in Table 4.

The amino acid compositions of DHPR from different sources are shown in Figure 1. Craine et al.[8] reported that the sheep liver enzyme contained one cysteine residue per subunit, which was not exposed in the native enzyme. On the other hand, Cheema et al.[17] reported that the sheep liver reductase contained one free cysteine and one cystine. The N-terminal residue of DHPR is blocked by acetylation in all the enzymes that were examined for N-terminal amino acids. In the beef and sheep liver DHPRs acetylisoleucine is probably the N-terminal amino acid residue for both enzymes.[17] The human brain reductase is also blocked at the N-terminal.[79]

1-5 Active site and mechanism of action

Dihydropteridine reductase forms a stable complex with NADH and multiple forms due to bound NADH have been reported by several authors (e.g. references 10, 11, and 76). These include unbound enzyme, NADH-bound enzyme subunit (1:1) and NADH-bound enzyme subunits (1:2). The stoichiometry of binding of this enzyme and cofactor was

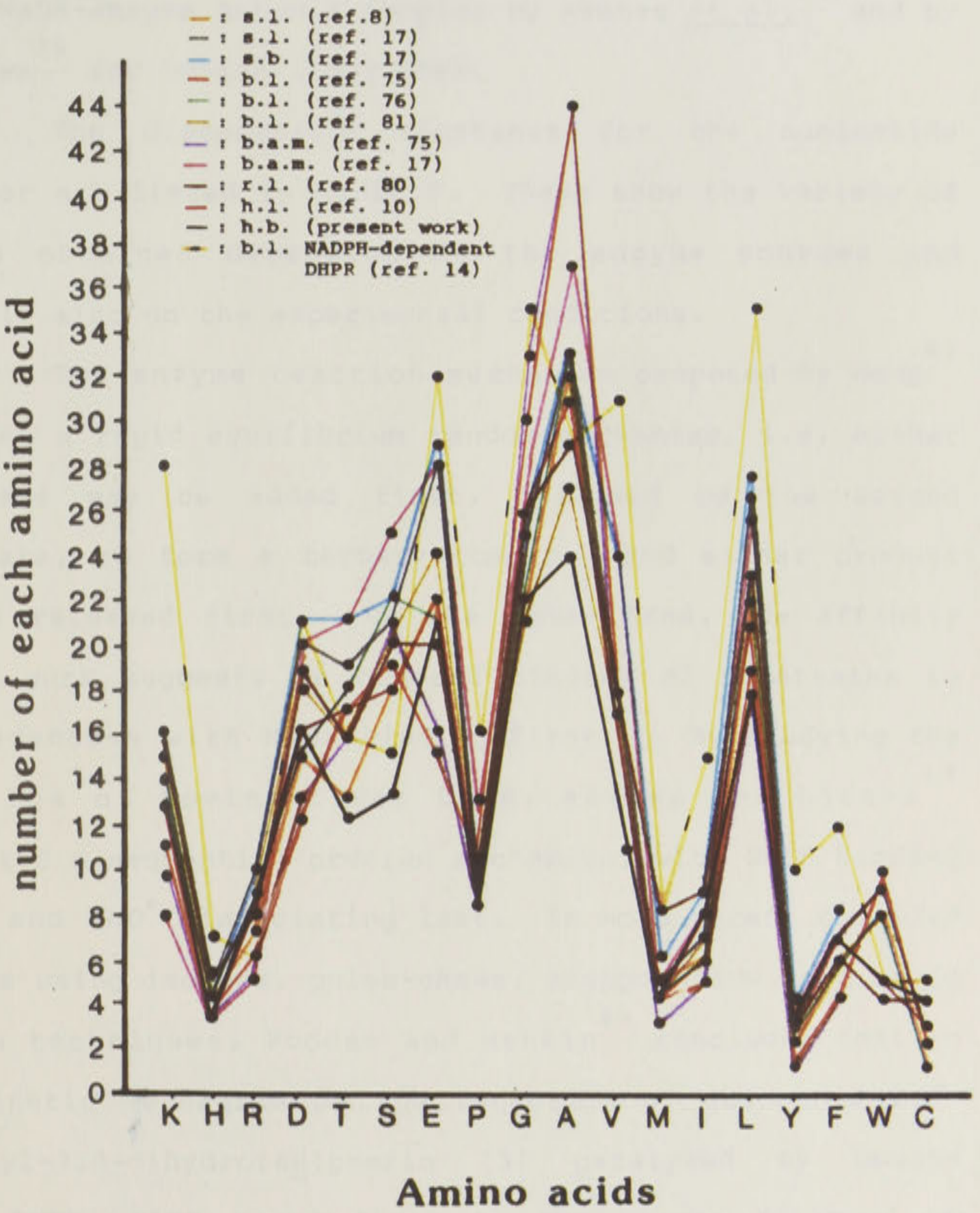


Figure 1. Amino acid compositions of DHPR from various sources. s.l. sheep liver, s.b. sheep brain, r.l. rat liver, b.l bovine liver, b.a.m. bovine adrenal medulla, h.l. human liver, h.b. human brain.

reported as being 1 : 2 NADH-enzyme subunits complex by Webber and Whiteley[82] for rat liver DHPR, and as being 1 : 1 NADH-enzyme subunit complex by Aksnes et al.[75] and by Hasegawa[76] for bovine liver DHPR.

The dissociation constants for the nucleotide cofactor are listed in Table 5. These show the variety of values obtained depending on the enzyme sources and probably also on the experimental conditions.

The enzyme reaction mechanism proposed by Wong[83] involves a rapid equilibrium random mechanism, i.e. either substrate may be added first, followed by the second substrate, to form a ternary complex, and either product may be released first. On the other hand, the affinity column work suggests an ordered binding of substrates to the reductase with NADH binding first.[81] By studying the kinetics of bovine liver DHPR, Aksnes and Ljones[18] suggested a sequential ordered mechamism, with NADH binding first and NAD^+ dissociating last. In more recent detailed studies using isotope, pulse-chase, stopped-flow, and rapid quench techniques, Poddar and Henkin[84] concluded that in the kinetic mechanism of the reduction of quinonoid 6,7-dimethyl-7,8-dihydro(6H)pterin [3] catalyzed by bovine liver DHPR, NADH bound first to the enzyme followed by quinonoid substrate, i.e. ordered mechanism. This ternary complex undergoes isomerization which is the rate determining step, followed by hydride transfer, and release of products. Randles[85] also proposed an ordered bi-bi mechanism for human brain DHPR by studying the temperature

Table 5. Dissociation constants for NADH and for quinonid 6-methyl-7,8-dihydro(6H)pterin.

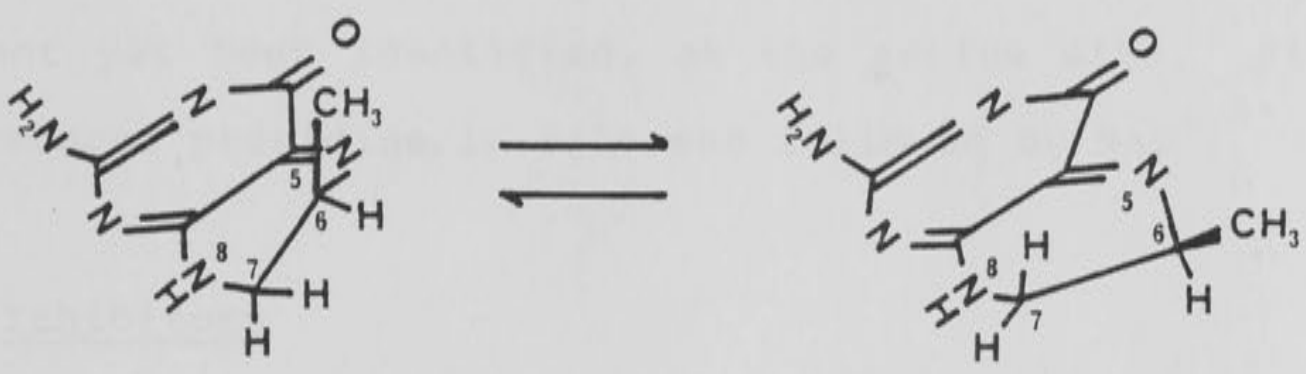
Enzyme	NADH (μM)	Quinonoid 6-MeDHP (μM)	ref.
r.l.	0.07 0.08*	2	86
	5.71 0.17*		82
b.l.	0.04 (at pH 7.8)		75
	0.01 (at pH 6.8)		75
b.a.m.	0.01 (at pH 6.8)		75

r.l. rat liver; b.l. bovine liver; b.a.m. bovine adrenal medulla.

* values which were re-calculated by using a computer programme (reference 22).

dependency of the enzyme activity.

Armarego et al.[47] suggested that the ethylene bridge joining N-5 to N-8 in the normal pterin substrate was not necessary for substrate activity, but this bridge did assist in binding at the enzyme active site. Armarego[87,88] also investigated the stereochemistry of quinonoid species at the active site by assaying the activity of quinonoid 6,6-dimethyl-7,8-dihydro(6H)pterin



axial equatorial

6-S-ENANTIOMER

and the activities of the two optical isomers of 6-methyl-5,6,7,8-tetrahydropterin. He concluded that an axial substituent at C-6 is not readily accepted by the reductase and that steric requirements at the active site around the C-7 position of the pterin are not as strict as those around C-6.[22] The pteridine substrate sits at the active site with the 6-alkyl substituent in the quasi equatorial conformation.[88]

^{1}H NMR revealed that the reduced pyrazine ring of the quinonoid species was in the half chair conformation and substituents at C-6 and C-7 could exist in axial or in equatorial conformations (cf. reference 73-b).

From the data available the chemical mechanism of the enzyme reaction can be summarized as follows: NADH binds to the enzyme followed by the pteridine cofactor with the 6-substituent in the equatorial conformation. Then a conformational change of the enzyme ternary complex at the active site occurs. This is followed by hydride transfer from the B face of NADH with transfer of the pro-S hydrogen[89] to N-5 of the quinonoid dihydropteridine, and then protonation of N-3 by some amino acid residue, which has not yet been identified, at the active site. Finally the reduced pteridine is released followed by NAD^+.[84]

1-6 Inhibitors

1-6-1 Pteridine analogues

The inhibition constants of DHPR by pteridine analogues are listed in Table 6. The inhibition appears to depend not only on the structure of the compound but also on the source of the enzyme. The assay conditions may also affect the inhibition (cf. Chapter 4, p 192). Substrate inhibition (i.e. quinonoid dihydrobiopterin) had been reported by Firgaira et al., and it occurred at substrate concentrations above 50 µM.[10]

Table 6. Inhibitions of DHPR by pteridine analogues.

Inhibitor	Enzyme	Type	Ki (μM)	ref.
Aminopterin	h.l.	non-comp.*	10	90
	h.l.	non-comp.#	11	90
	P.	comp.*	210	51
	s.l.	non-comp.*	20	17
	r.l.	comp.*	24.0	91
Amethopterin (Methotrexate)	s.l.	comp.*	40	8
	b.k.	mixed non-comp.*	30, 50 or 180	92
	b.k.	uncomp.#	40 or 70	92
p-Aminobenzoylglutamate	b.k.	comp.#	10,000 or 4,000	92
2,4-Diamino-6-methyl-pteridine	r.l.		inert	91
2,4-Diaminopteroic acid	r.l.	comp.*	29.5	91
2,4-Diamino-7,8-dihydro-pteroylglutamic acid	r.l.	comp.*	23.0	91
Quinonoid 2,4-diamino-dihydropteroylglutamic acid	r.l.	comp.*	22.0	91
Folic acid	s.l.	non-comp.*	240	17
6,7-Dimethylpterin	s.l.	non-comp.*	300	17
2,4-Diamino-6,7-dimethylpterin	s.l.	non-comp.*	80	17
2-Amino-6,7-dimethyl-pteridin-4(3H)-one	s.l.	non-comp.*	50	17

s.l. sheep liver; h.l. human liver; P. Pseudomonas; b.k. bovine kidney; r.l. rat liver; comp. competitive; non-comp. non-competitive;
* for substrate; # for NADH.

1-6-2 Thiol specific reagents

The inhibition constants of DHPR from various sources by thiol specific reagents and heavy metals are listed in Table 7. The table also shows the different degrees of inhibition (in percent) by reductase from different sources.

These data do not distinguish any one particular cysteine residue as necessary for enzyme activity, but indicate that thiol groups may be involved in the binding of the NADH cofactor to enzyme because of the protective effect of NADH against those inhibitions.

Table 7. Inhibition constants of DHPR by thiol specific reagents and metal complexes.

Inhibitor	Enzyme		ref.
Pb-acetate	r.b.	50μM(~50%)	93
Al^{3+}	r.b.	100μM(40%)	94
cis-$Pt(NH_3)_2Cl_2$	rat	170μM(28%)	95
PCMB	s.l.	1mM(>80%)	17
	h.l.	10μM(70%)	10
	r.l.	6molecule(100%)	80
DTNB	h.l.	100μM(60%)	10
	P.	570μM(~100%)	51
	r.l.	4 cysteine (80%)	80
NEM	h.l.	1mM	10
	P.	10mM(57%)	51
	r.l.	inert	80
IA	h.l.	10mM	10
IAA	r.l.	inert	80
$HgCl_2$	s.l.	10μM(>80%)	17
	h.l.	0.1μM(60%)	10
	P.	400μM(50%)	51

r.b. rat brain; c.l. chicken liver; s.l. sheep liver; h.l. human liver; *P.* *Pseudomonas*; r.l. rat liver.

IA iodoacetamide; IAA iodoacetic acid.

1-6-3 NADH analogues

The inhibition constants of beef DHPR by NADH analogues are listed in Table 8.

Table 8. Inhibition constants of DHPR by NADH analogues.

Inhibitor	enzyme	Type	Ki(μM)	ref.
AMP	b.l.	comp.	1,000	81
	b.k.	comp.	1,000	92
NAD^+	b.k.	comp.	300	92
Cibacron Blue	b.k.	comp.	0.3	92
Blue dextran	b.k.	comp.	0.3	92

b.l. bovine liver; b.k. bovine kidney.

1-6-4 Metabolites

Some of the reported inhibition constants of DHPR by amino acid metabolites and especially the catecholamine derivatives are listed in Table 9.

1-6-5 Quinone inhibitors

The reported inhibition constants of DHPR by compounds which possess quinone structures, and especially some drug metabolites, which are interesting from the pharmaceutical point of view, are listed in Table 10.

1-6-6 Antibodies

Antibodies which inhibit the activity of DHPR have

Table 9. Inhibition constants of DHPR by amino acid metabolites and their derivatives. Enzyme activities were assayed in the presence of peroxidase and hydrogen peroxide unless otherwise stated.

Inhibitor	Enzyme	Type	I_{50} (μM)	Ki (μM)	ref.
L-Phenylalanine	h.l.	non-comp.	>10,000		96
L-Tyrosine	h.l.		440		97
	h.l.	non-comp.	440	250	96
α-Methyltyrosine	h.l.		1,100		97
3-Iodotyrosine	h.l.		>2,000		97
Phenylpyruvic acid	h.l.	uncomp.*	6,500		90
	h.l.	non-comp.	4,700		96
	r.l.	mixed		50	98
L-Phenylacetic acid	h.l.	non-comp.	7,600		96
ρ-Hydroxyphenyl-pyruvic acid	h.l.	non-comp.	3.3	3.7	96
ρ-Hydroxyphenyl-acetic acid	h.l.		58	74	96
Tyramine	h.l.	non-comp.*	50		97
	h.l.	non-comp.#	24		97
	h.l.	non-comp.	40	50	96
Dopamine	r.l.		110		99
	h.l.	non-comp.*		6.5	90
	h.l.	non-comp.#		6.0	90
	h.l.	non-comp.*	14		97
	h.l.	non-comp.#	7		97
	h.b.	inertϕ			15
N-Methyldopamine	h.l.		27		97
3-O-methyldopamine	h.l.		60		97
4-O-Methyldopamine	h.l.		69		97
Homoveratrylamine	h.l.		10,000		97
5-Hydroxydopamine	h.l.		42		97
6-Hydroxydopamine	h.l.	non-comp.*		24	97
	h.l.	non-comp.#		24	97
(±)Octopamine	h.l.		190		97
Dopachrome	h.b.		600		15
Adrenochrome	h.b.	mixed*		29.8	15
				& 89	15
	h.b.	comp.#		4.9	15
L-Dopa	h.l.	non-comp.	230	260	96
Norepinephrine	r.l.	non-comp.		490	98
(-)-Norepinephrine	h.l.	non-comp.*		190	97
	h.l.	non-comp.#		160	97
(-)-Epinephrine	h.l.	uncomp.*		110	97
	h.l.	uncomp.#		140	97
3-O-methyl-epinephrine	h.l.		180		97

* for substrate; # for NADH: h.l. human liver; h.b. human brain; r.l. rat liver; ϕ enzyme activity was assayed in the absence of hydrogen peroxide.

Table 10. Inhibition constants of human liver DHPR by inhibitors which possess quinone structures. Enzyme activities were assayed in the presence of peroxidase and hydrogen peroxide after incubation of the inhibitor and the reductase.

Inhibitor	Type	I_{50} (μM)	Ki (μM)	ref.
17-β-Estradiol	non-comp.*		3.5	101
17-α-Estradiol	non-comp.	5.4		
Estriol	uncomp.*		3.3	
16-Epiestriol	non-comp.	0.9		
17-Epiestriol	non-comp.	1.9		
16,17-Epiestriol	non-comp.	1.1		
Estrone	non-comp.	8.0		
2-Hydroxyestradiol	non-comp.*		2.8	
2-Hydroxyestriol	non-comp.*		2.6	
2-Hydroxyestrone	non-comp.*		4.6	
2-Methoxyestradiol	non-comp.	5.4		
2-Methoxyestrone	non-comp.	7.9		
d-Equilenin	non-comp.	6.6		
Equilin	non-comp.	7.5		
4-Phenyl-[1]	non-comp.	12,000		102
1-Methyl-4-phenyl-[1]	non-comp.	3,000		
1-Methyl-4-(4'-chlorophenyl)-[1]	non-comp.	2,700		
4-(4'-Hydroxyphenyl)-[1]	non-comp.	5.9		
4-(3'-Methoxy-4'-hydroxyphenyl)-[1]	non-comp.	7.2		
4-(3',4'-Dihydroxyphenyl)-[1]	non-comp.	3.6		
1-Methyl-4-(3',4'-dihydroxyphenyl)-[1]	non-comp.	3.4		103
8-Amino-2-methyl-4-phenyl-[2]	non-comp.	150		
4-(4'-hydroxyphenyl)-[2]	non-comp.	45	35	
4-(3'-hydroxy-4'-methoxyphenyl)-[2]	non-comp.	241		
Maleic acid	inert			
R-(-)-Apomorphine	non-comp.	2.0	2.2	104
R-(-)-Norapomorphine	non-comp.	2.8		
R-(-)-N-n-Propyl-[3]	non-comp.	1.8	2.2*	
S-(+)-N-n-Propyl-[3]	non-comp.	2.2		
R-(-)-N-Chloroethyl-apomorphine	non-comp.	2.9		
R-(-)-N-Hydroxyethyl-[3]	non-comp.	1.0		
R-(-)-2,10,11-Trihydroxy-aporphine	non-comp.	1.6	1.9*	
R-(-)-2,10,11-Trihydroxy-N-n-propylnoraporphine	non-comp.	1.7		
R-(-)-Apocodeine	non-comp.	79		

* for substrate; # for NADH; [1] 1,2,3,6-tetrahydropyridine derivatives; [2] 1,2,3,4-tetrahydroisoquinoline derivatives; [3] norapomorphine derivatives.

been prepared for DHPR from different sources. The bovine brain antibody crossreacts with the enzyme from rat brain, rat kidney, and rat liver.[77] On the other hand, sheep liver antibody crossreacts with the enzyme from human, rat, and bovine tissues.[100]

The antibodies which are prepared for NADPH-dependent DHPR do not react with NADH-dependent DHPR and this method is used for the identification of the presence of NADPH-dependent DHPR in tissues.[11]

1-7 Aims

The aims of the work which is described in this thesis are directed to obtain information about the nature of the active site of human brain DHPR.

The amino acid residues that are essential for enzyme catalysis have not yet been identified. To obtain details of the chemistry of catalysis of DHPR, it is necessary to probe the active site. To achieve this it is at first important to obtain the amino acid composition of this enzyme. By using the results from the amino acid analysis, proper estimations of enzyme concentration are obtained; these are necessary for the study of the stoichiometry of NADH and the reductase.

The correct tautomer of quinonoid dihydropteridine species which is enzymically active is still not completely known. This has been studied in detail using the pteridine analogues where changes at C-2, C-4, C-6, N-8, and at the

2-amino group have been made, and is reported in this thesis. The results of such a study should be useful for structure-activity relationship which can give some idea of the space requirements and perhaps binding at the active site.

More information can be obtained about the ionic nature of the reactive amino acid residues at the active site by pH rate profile studies, and about the effect of the chemical structures of inhibitors. Such studies have been attempted. In the present work, platinum complexes have been examined as inhibitors which, with the aid of several thiol specific reagents, provided information regarding the reactivity of the cysteine residues in this enzyme. This study was carried out because it was known that several reductases have essential cysteine residues at the active site. These are apparently involved in the chemical mechanism of the enzyme reaction.

CHAPTER 2

AMINO ACID COMPOSITION, PROTEIN CONCENTRATION, SPECTROSCOPIC PROPERTIES AND REACTIVITY OF THIOL GROUPS

2-1 Introduction

Dihydropteridine reductases from various mammalian sources were isolated and their amino acid compositions were compared by several authors (see Chapter 1, Section 1-2, p 5).[22] In the case of the human reductase, Firgaira et al.[41] demonstrated that the enzymes from human liver, cultured fibroblasts and continuous lymphoid cells were immunologically identical, and concluded that these were encoded by the same structural gene(s). Several authors[17,75,76,81] reported that reductases from bovine liver and adrenal medulla had similar amino acid compositions but were not exactly the same. This might well be due to the differences in experimental methodology. Aksnes et al.[75] compared the enzyme from bovine liver and adrenal medulla under the same conditions and obtained the same amino acid composition for these two proteins. Sheep liver[8,17] and brain[17] DHPR were also compared and their amino acid compositions were the same. The human brain DHPR is most probably the same as the liver enzyme, because hyperphenylalaninemia patients which lack DHPR in liver and fibroblast tissues exhibit mental disturbance characteristic of DHPR deficiency. In an endeavour to obtain further data to establish the similarity of the human brain and liver enzymes the amino acid composition of the brain reductase has been determined and reported in this chapter.

In the previous publications, various authors used different methods for estimating protein concentration,

and this seriously affected studies of the stoichiometry of NADH and DHPR. In this work, the methods for estimating DHPR concentration were compared and then the stoichiometry of NADH binding was calculated using the correct protein concentration.

The spectroscopic properties of the enzyme are reported in the following sections. The reactivities of several thiol reagents towards human brain DHPR have also been investigated.

2-2 Enzyme purification

The isolation of DHPR from human brain was performed as described by Armarego and Waring[15] who used the naphthoquinone affinity chromatography technique of Cotton and Jennings.[105] The enzyme, dissolved in 50 mM Tris/HCl buffer containing 2 mM DTT, was stored in frozen aliquots at -20^0C and thawed when required. The enzyme used for the work described in this chapter, and in Chapter 4 was a partially purified fraction from human brain (after naphthoquinone affinity chromatography) kindly provided by Dr Armarego. It showed seven bands on SDS-PAGE in which the band at molecular weight ca 25,000 was the most intense (>90%).

The active fraction was purified further by passing through a DEAE-Sephadex A-50 column and eluting with a 0 - 250 mM KCl gradient in 50 mM Tris/HCl buffer. The active fractions eluted at 100 - 150 mM KCl and showed

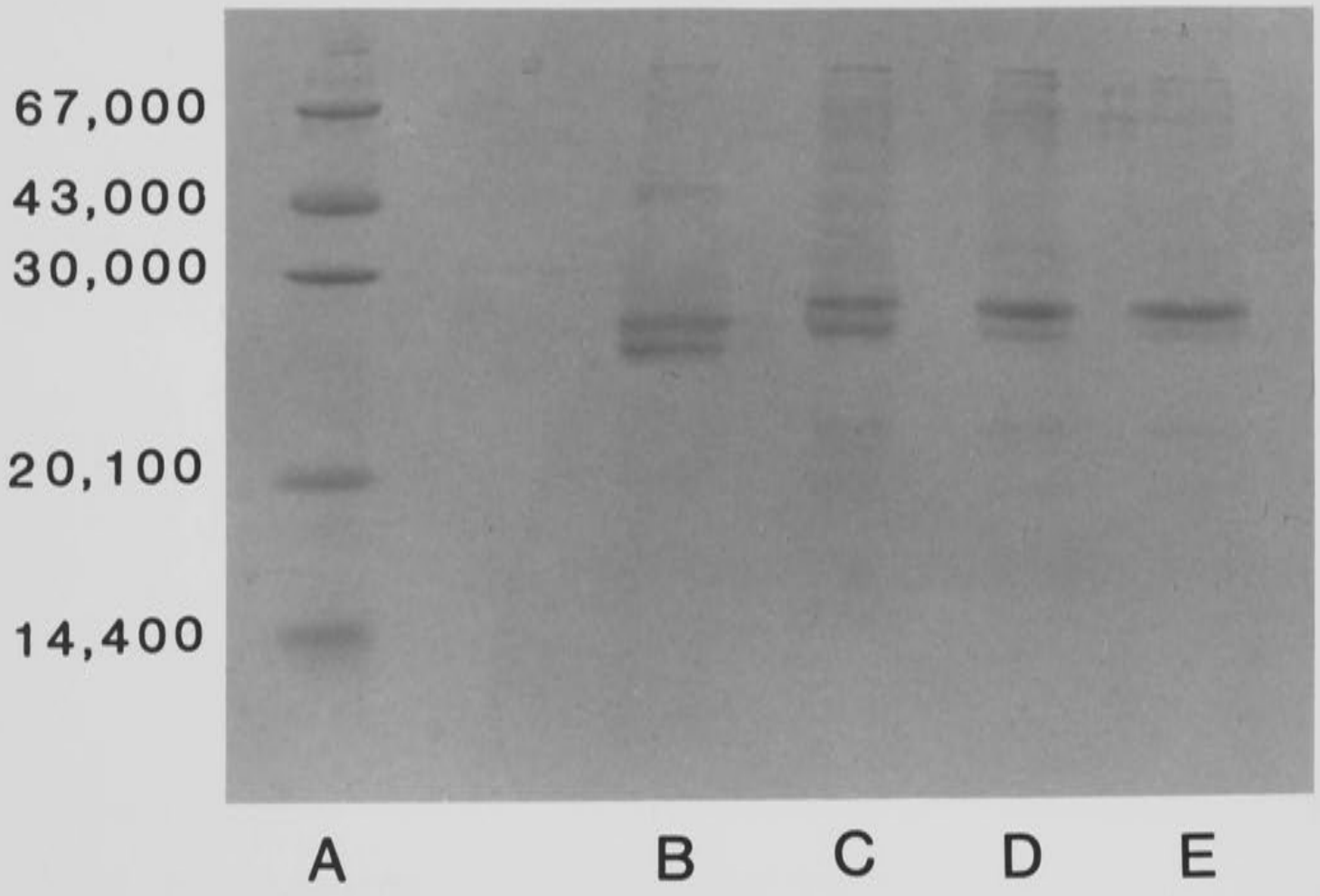


Figure 1. SDS-polyacrylamide gel electrophoresis of DEAE-Sephadex purified DHPR. Electrophoresis was performed on 15% of gel concentration at pH 9.4, 4^0C.
A: Low molecular weight standard (Pharmacia).
B: DHPR (4.3 µg, 1.2 nmol) with 1.2% SDS and 5.9% glycerol (no incubation).
C: DHPR (4.3 µg) was incubated with 0.6% SDS, 3% glycerol and 1.2% β-mercaptoethanol for 30 min at 100^0C.
D: DHPR (4.3 µg) was incubated as above for 5 min.
E: DHPR (4.3 µg) with 0.6% SDS, 3% glycerol and 1.2% β-mercaptoethanol (no incubation).
The gel was stained by Serva blau R-250.

monomer and dimer molecular weight bands on SDS-PAGE and PAGE respectively. On SDS-PAGE, in the absence of mercaptoethanol or when heated in 0.1% SDS and 1% mercaptoethanol at 100^0C, for 30 min, this enzyme showed two close bands around Mr 26,000 ± 1,000. With mercaptoethanol, on the other hand, only one band close to the slower moving band of the previous two bands was observed (Figure 1). It appears that the formation of two bands may be associated in some oxidation of cysteine residues.

The purified DHPR was stored in 50 mM Tris/HCl buffer (pH 7.3) or in 50 mM potassium phosphate buffer (pH 7.2) with 2 mM DTT and kept in glass vials in 1 ml aliquots. When DTT and NADH free enzyme was required it was subjected to prolonged dialysis. However, the thawed enzyme (stock enzyme), which apparently contained only one protein, gave a small amount of white precipitate on dialysis against the above respective buffers at 4^0C. The amount of precipitate was ca 30% of the total protein, but the total enzyme activity of stock DHPR was the same as the one of the dialysate, implying that the precipitated protein was essentially inactive enzyme.

This dialyzed enzyme was used for most of the work in the present chapter and in Chapter 4 unless otherwise stated.

2-3 Amino acid composition

A standard procedure[106] was used to determine the amino acid composition of the proteins. The proteins were carboxymethylated with iodoacetic acid and hydrolyzed with 6 M HCl. The amino acid compositions were determined (duplicated runs) using a Beckman Systems 6300 HPLC analyser. No trace of cystine residues was observed in the resulting chromatography, i.e. alkylation was complete. Cysteine was also alternatively determined by oxidation of the protein with performic acid and estimated as cysteic acid using the same analyser. Bovine ß-lactoglobulin B, kindly supplied by Dr D.C. Shaw[107] was used as standard in order to check the techniques and equipment used. Tyrosine residues could be obtained by these methods but tryptophan residues were destroyed. The tryptophan residues were determined using Goodwin and Morton's and also Edelhoch's UV methods (modified) (see below).[108]

The amino acid compositions were calculated using a monomer molecular weight of 25,000 Daltons for DHPR, and 18,000 Daltons for ß-lactoglobulin B and are shown in Table 1.

The amino acid ratios in Table 1 were based on the assumption that the determined concentration of alanine was correct, because this is one of the most stable amino acids in the analytical procedures used. The UV estimation of the tryptophan to tyrosine ratio in human brain DHPR was 1.8 ± 0.2. The number of tyrosine residues

Table 1. Amino acid compositions.

	Human[a] brain DHPR	Human[b] liver DHPR	Human[c] liver DHPR	β-LG[d]	β-LG[e]
Ala	24	31	27	15	15
Cys	4[f] (3)[g]	3[h]	3	5	5
Asp	16	16	14	15	15
Thr	12(13)	17	17	8	8
Ser	13	18	18	7	7
Glu	21	22	22	25(26)	25
Pro	8(9)	9	9	8	8
Gly	22	26	26	6	4
Val	13	17	17	7	9
Met	4	4	4	4	4
Ile	8	8	8	8(9)	10
Leu	18	21	19	22	22
Tyr	3	3	3	3(4)	4
Phe	7(8)	7	7	4	4
His	3	5	4	2	2
Lys	13	14	14	15	15
Arg	9	9	8	3	3
Trp	5(6)	5	5	2	2
calculated Mol. Wt.	25,400 (25,800)	29,000	25,600	20,800 (21,200)	21,200

a : compositions based on Mr values of 25,000 (present work)
b : reference 10
c : recalculated from reference 3 on the basis of Mr 25,000 as a monomer molecular weight
d : this work, β-LG = bovine β-lactoglobulin B
e : reference 107
f : determined as cysteic acid
g : determined by the reaction with PCMB or DTNB
h : determined as S-carboxymethylcysteine

per subunit was three from the amino acid analysis, so the number of tryptophan residues were 5 ~ 6. The analysis for ß-lactoglobulin B showed that the amino acid recovery from the analyzed data was in a very good agreement with the amount of protein used (95 ~ 102%). From this recovery, the DHPR concentration of this dialyzed enzyme solution was estimated to be 247.6 (± 1.6) µg/ml from two estimates.

Although there were small discrepancies between amino acid compositions of human brain and liver reductases, these were within the experimental error of the method. The true amino acid composition will be revealed when the complete sequence has been determined.

2-4 Estimation of the protein concentration

Protein concentrations have been measured in several ways by different workers. The general methods for this purpose are summarized in Table 2 (cf. reference 109).

A reliable method was required for obtaining the concentration of DHPR in the present study and it was necessary to find out which rapid method (e.g. Bio-Rad, Lowry) gave correct protein concentrations. In this work the protein concentration from the amino acid analysis method (which is the most reliable) has been compared with those from the Bio-Rad and Lowry assays in order to find out if they gave reliable values. Also, once the protein concentration was established for the pure enzyme then the extinction coefficient of DHPR at 280 nm can be determined

Table 2. General methods for determining protein concentration.

methods	principle	limit(mg)	interference	comments
Dry weight	Dry weight of purified protein	1	———	variable content of water
Kjeldahl	Measures NH_3 derived from nitrogen in the protein	1.5×10^{-3}	nitrogen compounds e.g. $(NH_4)_2SO_4$	several methods can be used for determination of NH_3
Biuret	Reaction between Cu^{2+} and peptide bonding in alk. solution	1	EDTA, Tris, Amide, $(NH_4)_2SO_4$	low sensitivity, requires arbitrary standards
Lowry	Reaction between phenol reagent and Tyr, Trp, and Cys and the Biuret reaction were mixed	3×10^{-3}	reducing reagents, EDTA, phenol, Triton X-100, amino acids, glycerol	large differences between protein molecules, requires arbitrary standards
Pigment binding	In acidic solution, the complex from protein and Coommassie blue G-250 gives blue colour	1×10^{-3}	Triton X-100(>0.1%), SDS(>0.1%), DNA(>0.1%), etc.	as above
Amino acid analysis	Acid hydrolysis and amino acid analysis by HPLC	50 pmol	amines	requires prior knowledge of approx. Mr
UV (280nm)	Measurement of absorption at ca 280 nm from Trp and Tyr residues	0.1	nucleic acids, aromatic compounds, etc.	large differences between proteins, requires pure protein and ε values
Fluorescence	Titration by specific reagents to give fluorescence after binding	5×10^{-5}	peptides, amino acids	sensitive, requires pure protein and Mol. Wt.

accurately and later used to estimate DHPR concentrations of pure enzyme preparations.

The pigment binding method using the Bio-Rad micro assay[110] and the Lowry method[111] are commonly used. The Bio-Rad method is reasonably sensitive, easy to perform, and gives results in the shortest time. Like the Lowry method, calibration with a standard protein is neccessary and bovine serum albumin is the most popularly used standard. This method can give incorrect concentrations because of impurities or the nature of protein itself (see Table 2). The Lowry method was developed by Lowry et al. in 1951 and has been used in many laboratories, although like the Bio-Rad method, it can give quite large differences in values depending on the protein and can be out by a large factor, e.g. of two or three.

If the recovery from the amino acid analysis is higher than 90%, this method gives results that are almost as reliable as the dry-weight method, and requires much less protein. The protein concentrations of DHPR (DTT free or in 2 mM DTT) determined by the various methods are compared in Table 3.

The results from the amino acid analysis and the Bio-Rad micro assay are in quite good agreement for human brain DHPR. The Lowry method gave almost twice the value obtained by the above methods for human brain DHPR. It can be concluded that for routine protein determinations the Bio-Rad assay will give more reliable values for human brain DHPR.

Table 3. Protein concentrations by the different estimations.

method	protein	protein concentration (µg/ml)
a	DHPR (DTT free)	262
b	DHPR (DTT free) DHPR (2mM DTT)	500 583
c	DHPR (DTT free)	248
d	DHPR (DTT free)	255

a: Bio-Rad micro assay

b: Lowry method

c: Amino acid analysis

d: UV absorption using ε_{280} = 90,000 $M^{-1}.cm^{-1}$ in buffer (pH 7.2)

2-5 UV and fluorescence spectra

Dihydropteridine reductase from human brain has strong UV absorption like DHPR from other sources, i.e. it possesses a wavelength maximum around 280 nm with a shoulder at 290 nm (see Figure 2). The extinction coefficient of 92,800 $M^{-1}.cm^{-1}$ at 280 nm, pH 7.20 (0.1 M potassium phosphate buffer) determined in this work is based on the protein concentration obtained from the Bio-Rad assay. This value is in good agreement with the ε_{280} values of DHPR from other sources. For very pure preparations of enzyme, the UV method is quite sensitive for determining protein concentrations. Thus for an ε_{280} value of 92,800, 54 µg/ml of DHPR will give 0.1 absorbance units for a 1 cm cell.

The fluorescence emission spectra of dialyzed DHPR (DTT free) were measured at 20^0C. The excitation wavelength of 280 nm affected both the tyrosine and the tryptophan residues and gave much stronger emission spectra than when excitation was at 290 nm. In proteins containing all three aromatic amino acids, phenylalanine, tyrosine and tryptophan, the observed emission spectrum is due mainly to the tryptophan residues (in neutral aqueous solutions, the emission maxima of tyrosine and tryptophan residues are at about 308 nm and 348 nm respectively).[112] When the emission spectrum (excitation at 280 nm) of human brain DHPR was scanned from 310 nm to 550 nm (Figure 3), the emission maximum was at 351 nm (compare with the emission

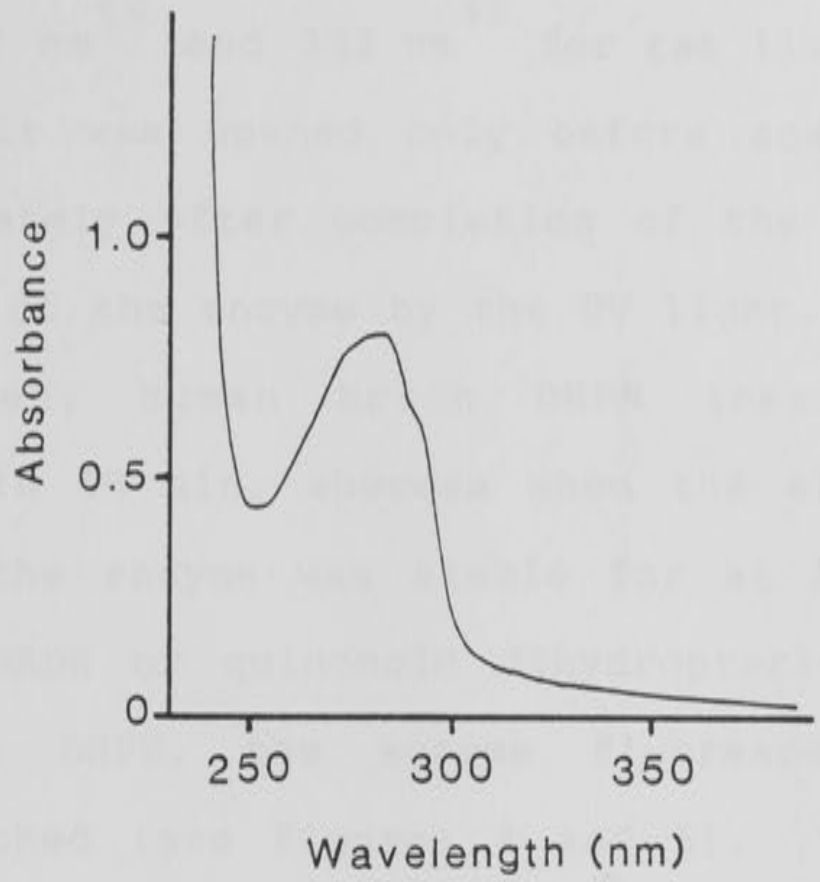


Figure 2. UV spectrum of DHPR (2 mM DTT in 50 mM potassium phosphate buffer, pH 7.2).

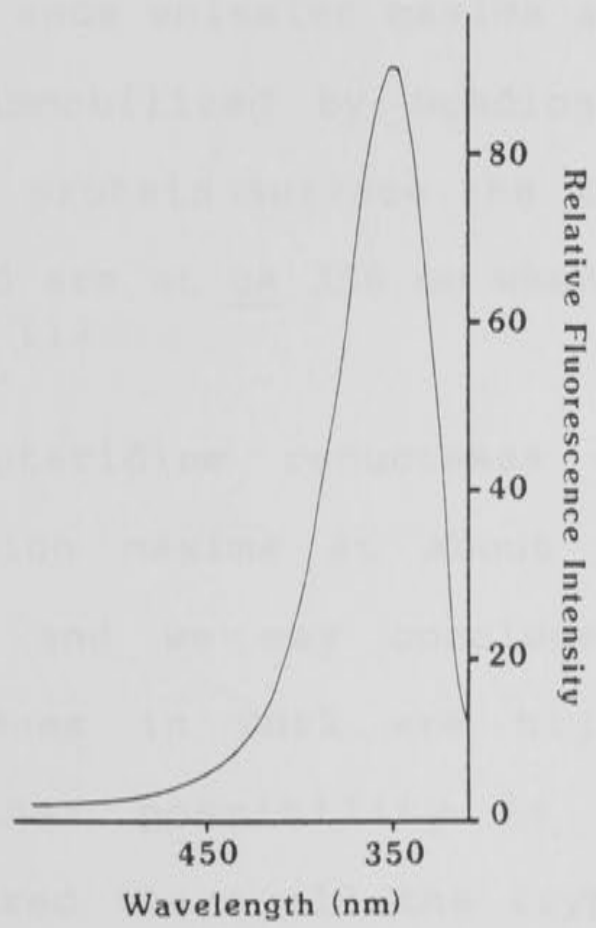


Figure 3. Fluorescence emission spectrum of DHPR. DHPR (DTT free, 10 µl, 68 pmol) was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) (490 µl) and excited at 280 nm (slit 5 nm) at 20^0C.

maxima at 350 nm[86] and 335 nm[82] for rat liver DHPR). The excitation slit was opened only before scanning and was closed immediately after completion of the scan to avoid deterioration of the enzyme by the UV light. If this slit was kept open, human brain DHPR lost 11% of its fluorescence in 50 min, whereas when the slit was opened occasionally the enzyme was stable for at least 5.5 h at 20^{0}C. When NADH or quinonoid dihydropteridine substrate was bound to DHPR, the enzyme fluorescence was very strongly quenched (see Figures 4 and 5). The decreasing fluorescence with time in Figure 5 was due to the rearrangement of the quinonoid pterin.

In general, fluorescence emission spectra of proteins in which the tryptophan residues are buried in the protein molecule show emission maxima at ca 330 nm, whereas when they are immobilized by bonding (e.g. hydrophobic, hydrogen) at the protein surface the maxima are shifted to about 340 nm, and are at ca 350 nm when they are completely exposed to water.[113]

Dihydropteridine reductases from other sources also have emission maxima at about 350 nm (except for reference 106), and we may conclude that most of the tryptophan residues in DHPR are highly exposed to the solvent. Another possibility is that DHPR used is partially denatured where all the tryptophan residues are exposed to the solvent. However, the DHPR used in this work was not denatured.

On denaturation the emission wavelength maximum

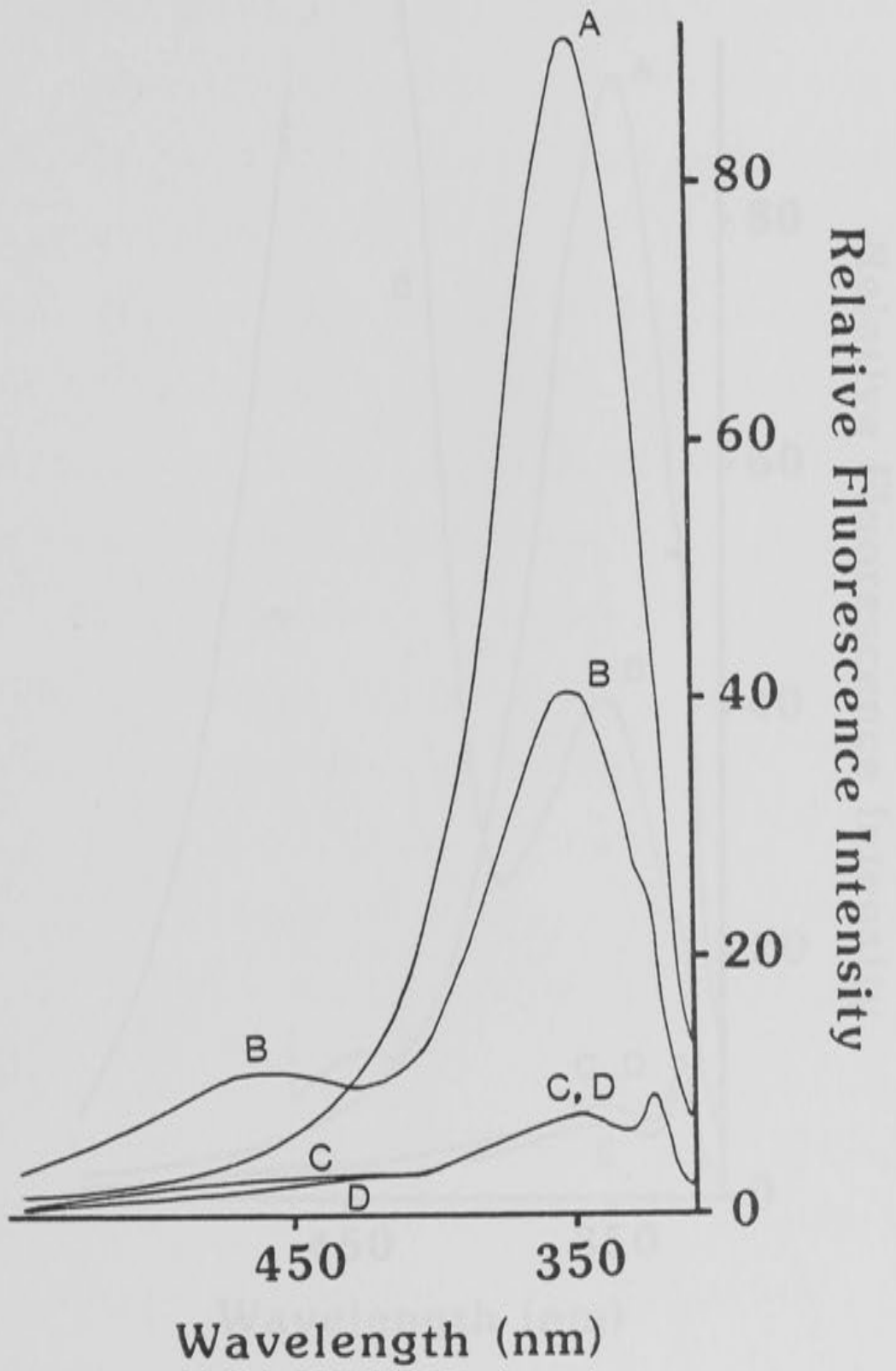


Figure 4. Effect of NADH on the fluorescence spectrum of DHPR at pH 7.2, 20^{0}C. A: enzyme (0.14 µM) in buffer (pH 7.2); B: enzyme (0.14 µM) + NADH (0.8 µM) in buffer; C: NADH (0.8 µM) in buffer; D: 0.1 M phosphate buffer (pH 7.2).

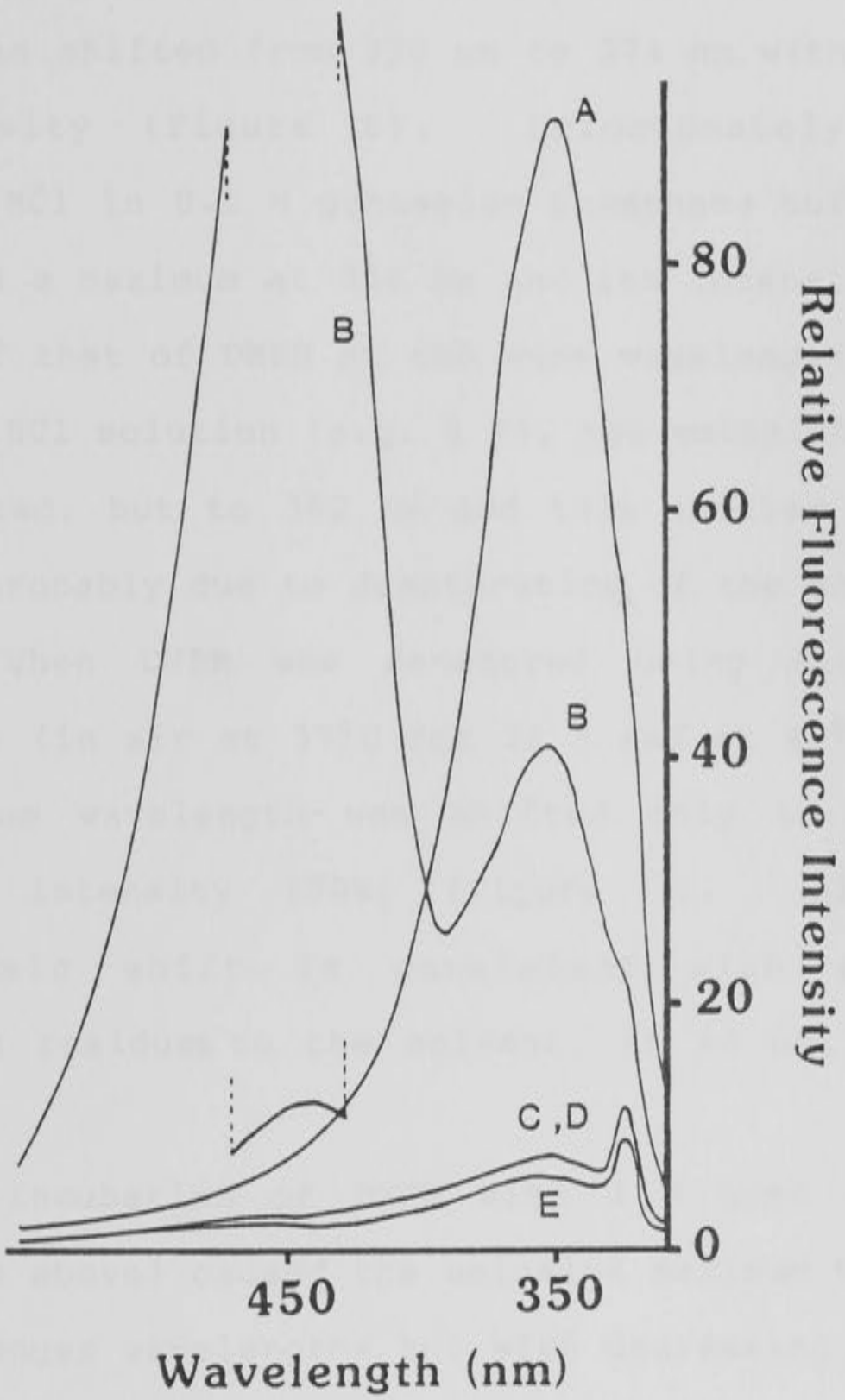


Figure 5. Effect of quinonoid 6-methyl-7,8-dihydro(6H)-pterin on the fluorescence spectrum of DHPR at 20°C.
A: DHPR (0.14 µM) in 0.1 M phosphate buffer; B: enzyme + quinonoid 6-MeDHP [generated by mixing of 6-MeTHP (in 4 mM HCl, 43 µM) and $K_3Fe(CN)_6$ (100 µM)]; C: $K_3Fe(CN)_6$ (100 µM) with 4 mM HCl in buffer; D: $K_4Fe(CN)_6$ (100 µM) with 4 mM HCl in buffer; E: 0.1 M potassium phosphate buffer (pH 7.2).

should be shifted to longer wavelength. When human brain DHPR was denatured with 8 M guanidine.HCl, the wavelength maximum was shifted from 350 nm to 374 nm without decrease in intensity (Figure 6). Unfortunately, the 8 M guanidine.HCl in 0.1 M potassium phosphate buffer (pH 7.2) itself had a maximum at 384 nm and its intensity at 370 nm was 78% of that of DHPR at the same wavelength. In weaker guanidine.HCl solution (e.g. 4 M), the emission maximum was also shifted, but to 362 nm and this smaller bathochromic shift is probably due to denaturation of the enzyme.

When DHPR was denatured using strong thermal conditions (in air at 37^0C for 24 h and at 45^0C for 21 h), the maximum wavelength was shifted only to 362 nm with decreased intensity (90%) (Figure 7). Although this bathochromic shift is consistent with exposure of tryptophan residues to the solvent, it is not as large as expected.

Incubation of DHPR with 4 M urea in phosphate buffer (as above) caused the emission maximum to shift with time to longer wavelengths but with decreasing fluorescence intensity (0 h, 352 nm, 100% ; 2.5 h, 357 nm, 78% ; 13 h, 357 nm, 59%) (Figure 8). (Either an increase or decrease had been observed previously upon urea denaturation depending on the protein.[114]) 4 M Urea itself had an emission maximum at 437 nm due to impurities but this was too weak to interfere in the previous experiment. Theoretically the fluorescence intensity of the protein should increase on denaturation and the observed decrease

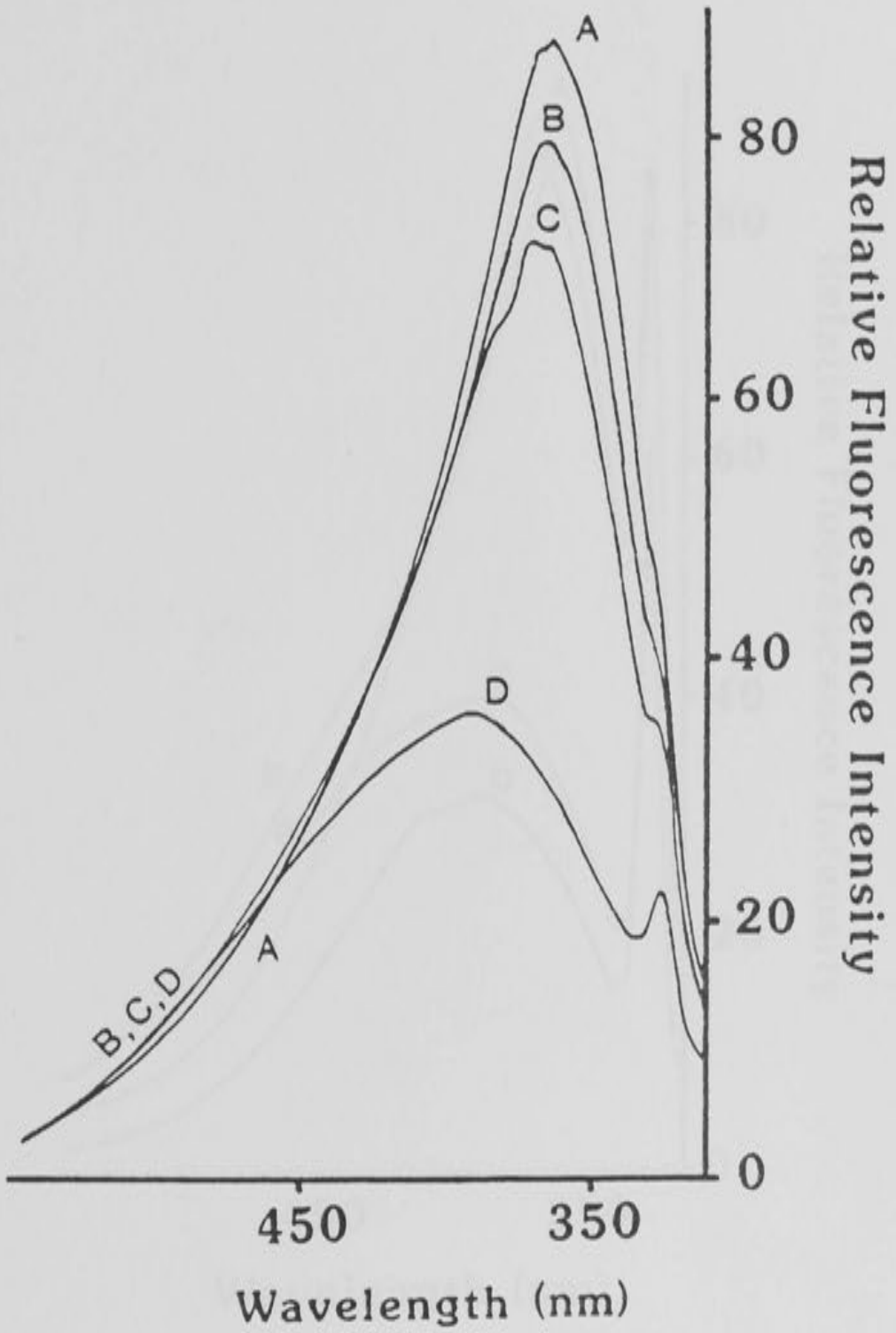


Figure 6. Fluorescence spectra of guanidine hydrochloride-denatured DHPR at 20°C. A: DHPR (0.07 μM) in 4 M guanidine.HCl in 0.1 M phosphate buffer (pH 7.0); B: NADH (0.8 μM) was added to above solution; C: after 15 min from NADH addition; D: NADH (0.8 μM) in 4 M guanidine.HCl solution (pH 7.0).

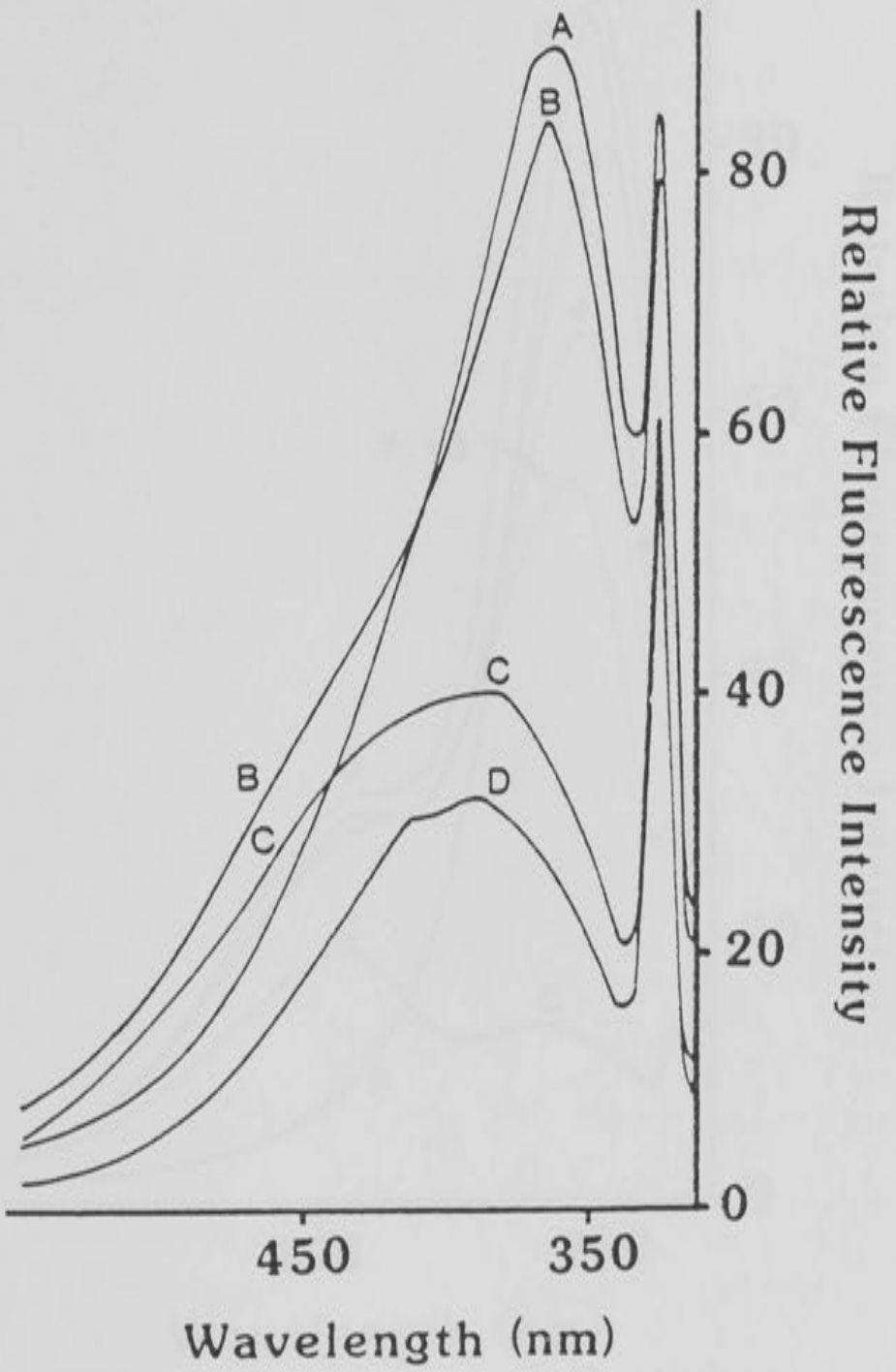


Figure 7. Fluorescence spectra of thermally denatured-DHPR at 20^{0}C, pH 7.2. A: Thermally denatured by heating (at 37^{0}C for 24 h and 45^{0}C for 21 h) DHPR (0.14 µM) in 0.1 M phosphate buffer (pH 7.2); B: NADH (0.8 µM) was added to above enzyme solution; C: NADH (0.8 µM) in 0.1 M phosphate buffer (pH 7.2); D: 0.1 M phosphate buffer (pH 7.2)

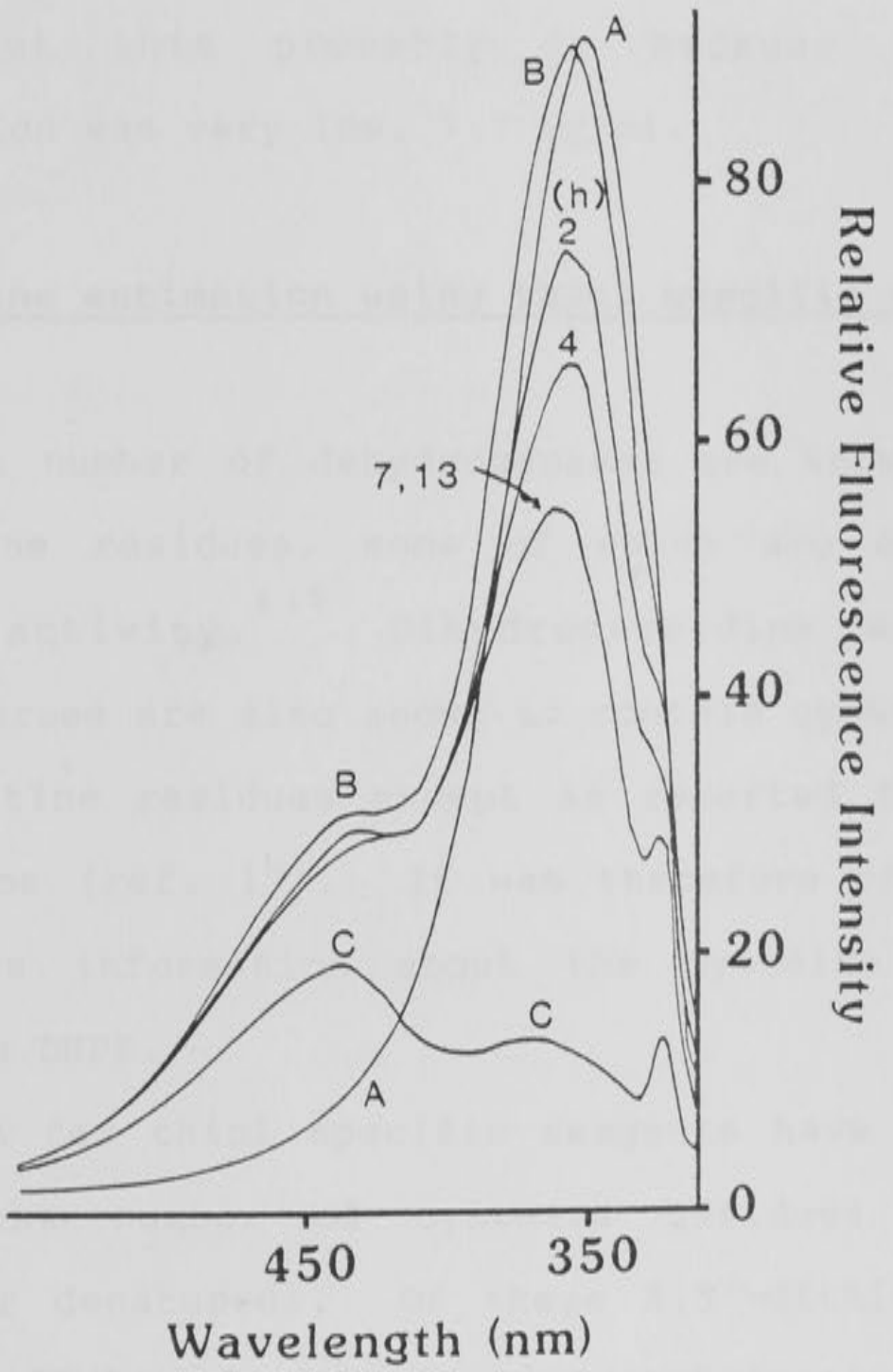


Figure 8. Fluorescence spectra of urea denatured DHPR at 20°C, pH 7.6. A: DHPR (0.07 µM) in 0.1 M potassium phosphate buffer (pH 7.2); B: DHPR (0.07 µM) in 4 M urea (pH 7.6), times after mixing (h); C: 4 M urea in 0.1 M phosphate buffer (pH 7.6).

in intensity on thermal denaturation can be explained by preferential adsorption of the denatured protein onto the wall of the cuvette. No turbidity was observed in the cuvette but this probably is because the protein concentration was very low, 7.7 µg/ml.

2-6 Cysteine estimation using thiol specific reagents

A number of dehydrogenases are known to contain few cysteine residues, some of which are essential for catalytic activity.[115] Dihydropteridine reductases from various sources are also known to contain cysteine residues but no cystine residues except as reported for the sheep liver enzyme (ref. 17). It was therefore of interest to obtain more information about the cysteine residues in human brain DHPR.

A few thiol specific reagents have been used to estimate the number of cysteine residues in proteins (natured or denatured). Of these 5,5'-dithiobis(2-nitro-benzoate) (DTNB), 2-nitro-5-thiocyanobenzoate (NTCB), and p-chloromercuribenzoate (PCMB) have been used in the present work in order to obtain some information about the nature of the thiol groups in human brain DHPR. It is possible to titrate thiol groups spectroscopically by using those reagents.

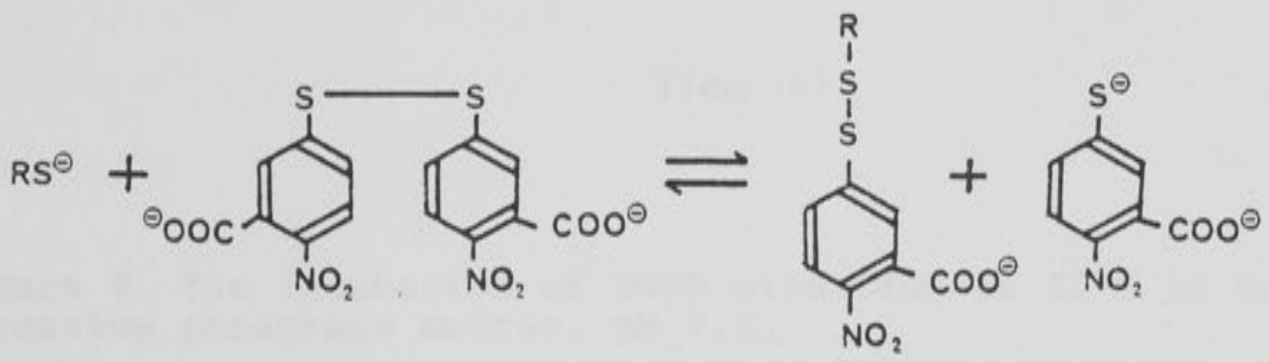
DTNB interacts primarily with the more reactive thiol groups, i.e. highly exposed free cysteine residues, probably because of its molecular size. PCMB and NTCB, on

the other hand, react with further cysteine residues which are located inside of molecule.

2-6-1 Reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)

Although DTNB[116] can react only with highly active cysteine residues under non-denaturing conditions, it does react with more cysteine residues in the denatured protein.

When the protein solution is incubated with an excess of DTNB, thionitrobenzoate anion (thiolate anion, TNB) is liberated, and the reaction is followed by measuring the absorbance at 412 nm of the thiolate anion. Excess of DTNB is necessary to minimize the reverse reaction (Scheme 1). The blank experiment is carried out



Scheme 1

with the corresponding buffer solution and a typical result is shown in Figure 9.

The concentration of cysteine residues which react with DTNB is the same as the concentration of TNB formed and is calculated from following equation (1),

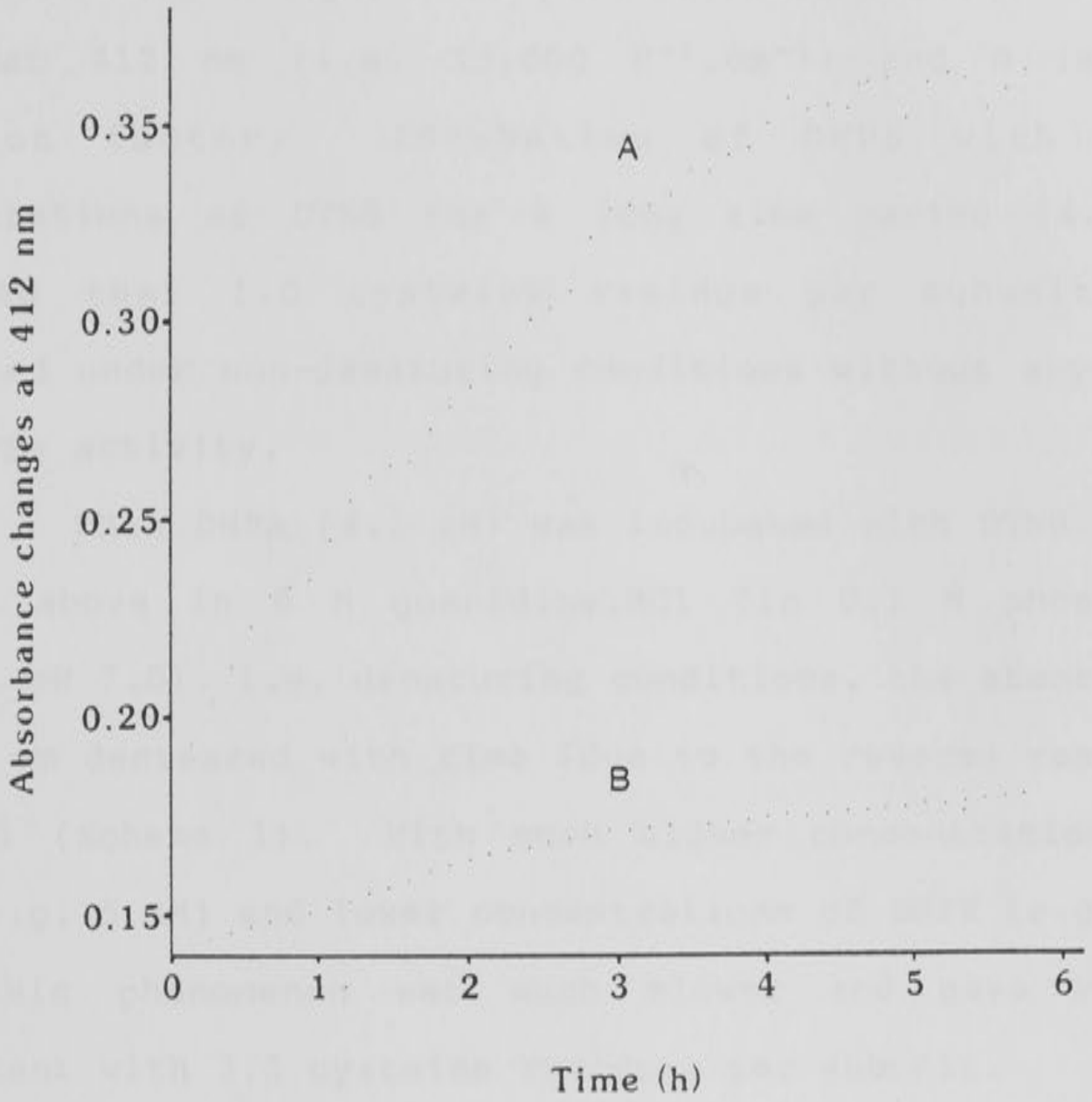


Figure 9. The incubation of DHPR with DTNB at 21^{0}C in 0.1 M potassium phosphate buffer, pH 7.2.

A: enzyme (DTT free, 150 µl, 9.96 µM) was incubated with 10 mM DTNB (30 µl, 1.07 mM) in 0.1 M phosphate buffer (pH 7.2) (100 µl) for 6 h.

B: Blank [0.1 M Phosphate buffer (pH 7.2) (250 µl) was incubated with 10 mM DTNB (30 µl, 1.07 mM).]

$$C = \frac{A}{\varepsilon} \times D \quad \text{——— (1)}$$

where C is cysteine concentration in the original solution, A is absorbance change, ε is extinction coefficient of TNB anion at 412 nm (i.e. 13,600 $M^{-1}.cm^{-1}$) and D is the dilution factor. Incubation of DHPR with high concentrations of DTNB for a long time period (4.5 h) revealed that 1.0 cysteine residue per subunit was estimated under non-denaturing conditions without any loss of enzyme activity.

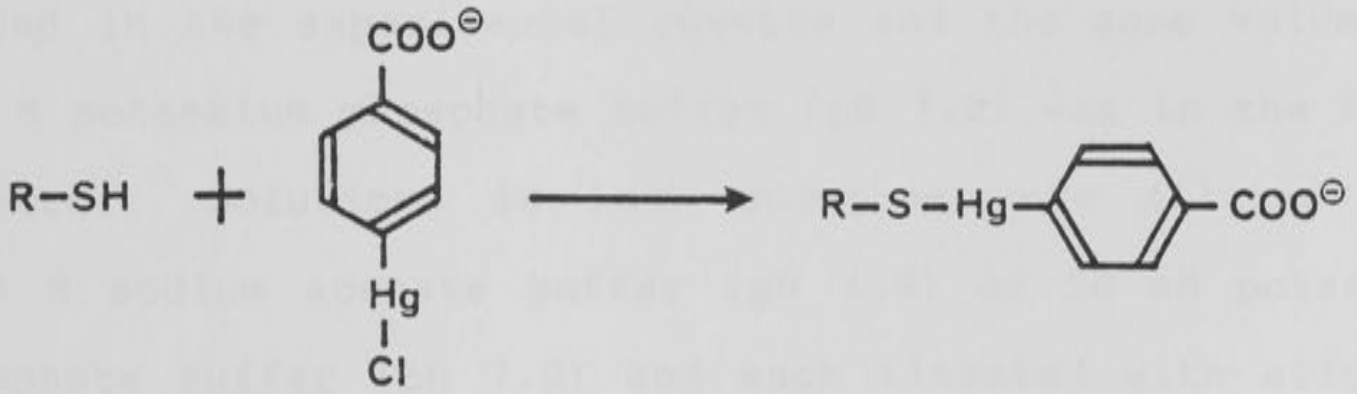
When DHPR (4.2 μM) was incubated with DTNB (0.24 mM) as above in 8 M guanidine.HCl (in 0.1 M phosphate buffer, pH 7.0), i.e. denaturing conditions, the absorbance at 412 nm decreased with time (due to the reverse reaction by TNB) (Scheme 1). With much higher concentrations of DTNB (e.g. 5 mM) and lower concentrations of DHPR (e.g. 2.1 μM), this phenomenon was much slower and gave values consistent with 3.5 cysteine residues per subunit.

When DHPR (5.1 μM) was pre-incubated with NADH (286 μM) for 10 min prior to titration with DTNB, 1.4 -SH per subunit (i.e. one -SH group per subunit) reacted with DTNB with ca 34% enhancement of enzyme activity. This enhancement of enzyme activity may be explained by a conformational change at the active site due to the reaction of DTNB at a remote site which affects the binding of substrates and/or release of products. From the above observations, human brain DHPR seems to have one free cysteine residue on the outer face of the enzyme and the

resulting partial conformational changes after reacting with DTNB do not decrease the enzyme activity.

2-6-2 Reaction with p-chloromercuribenzoic acid (PCMB)

p-Chloromercuribenzoic acid (PCMB)[117,118] is the most widely used organo-mercuric compound for the estimation of cysteine residues in proteins. It is not as toxic as the previously used inorganic mercury[117] and the formation of p-carboxyphenylmercaptide can be detected at 255 nm (pH 4.6) or 250 nm (pH 7.0) (Scheme 2). In acidic



Scheme 2

solution (pH 4.6), PCMB reacts with exposed and internal -SH groups in the protein. PCMB in neutral buffer (pH 7.0), on the other hand, reacts with the more exposed -SH groups as in the case of DTNB and also with some of internal -SH groups which are not so deeply buried in the protein structure.

The stock solution of PCMB was made by dissolving the acid into 1.6 mM NaOH and aliquots were re-mixed with 50 mM phosphate buffer (pH 7.0) or 0.33 M sodium acetate

buffer (pH 4.6). In this stock solution, p-hydroxymercuri-benzoate was formed, because of the higher affinity of hydroxide ions for the mercury than chloride ions (1,000 times). The proportion of these two components in the solutions depended on the concentration of chloride ions and pH. Because precipitation of white solids occurred on standing, solutions (of pH 7.0 and 4.6) were centrifuged and the concentrations of PCMB in the supernatants were estimated with glutathione before use. The titration was followed using a double beam UV spectrometer at 25^0C for both the acidic and neutral conditions.

The protein solution (DTT and NADH free) was placed in the experimental cuvette and the same volume of 0.1 M potassium phosphate buffer (pH 7.2) was in the blank cuvette. Solutions in both cuvettes were diluted with 0.33 M sodium acetate buffer (pH 4.6) or 50 mM potassium phosphate buffer (pH 7.0) and each titrated with aliquots of the respective standardized PCMB solutions. A typical curve is shown in Figure 10. The concentration of -SH groups was estimated from the concentration of PCMB at the intersection of the two lines. The number of -SH groups per subunit estimated in sodium acetate buffer (pH 4.6) was 2.7. Titration in 2% SDS solution in the same buffer gave 2.8 -SH per subunit value.

The reaction was much slower at pH 7.0, because it depended on the reactivity of each -SH group in the protein. The DHPR enzyme activity was also measured at the same time by assaying aliquots from the cuvettes (Figure 11).

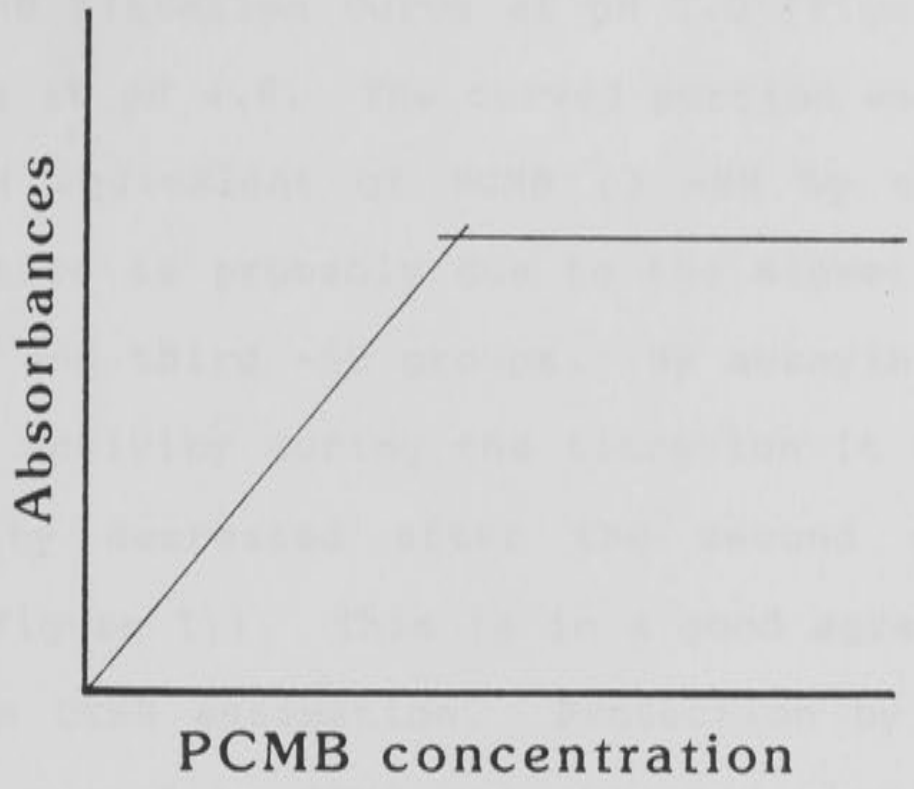


Figure 10. Typical PCMB titration curve (cf. reference 117).

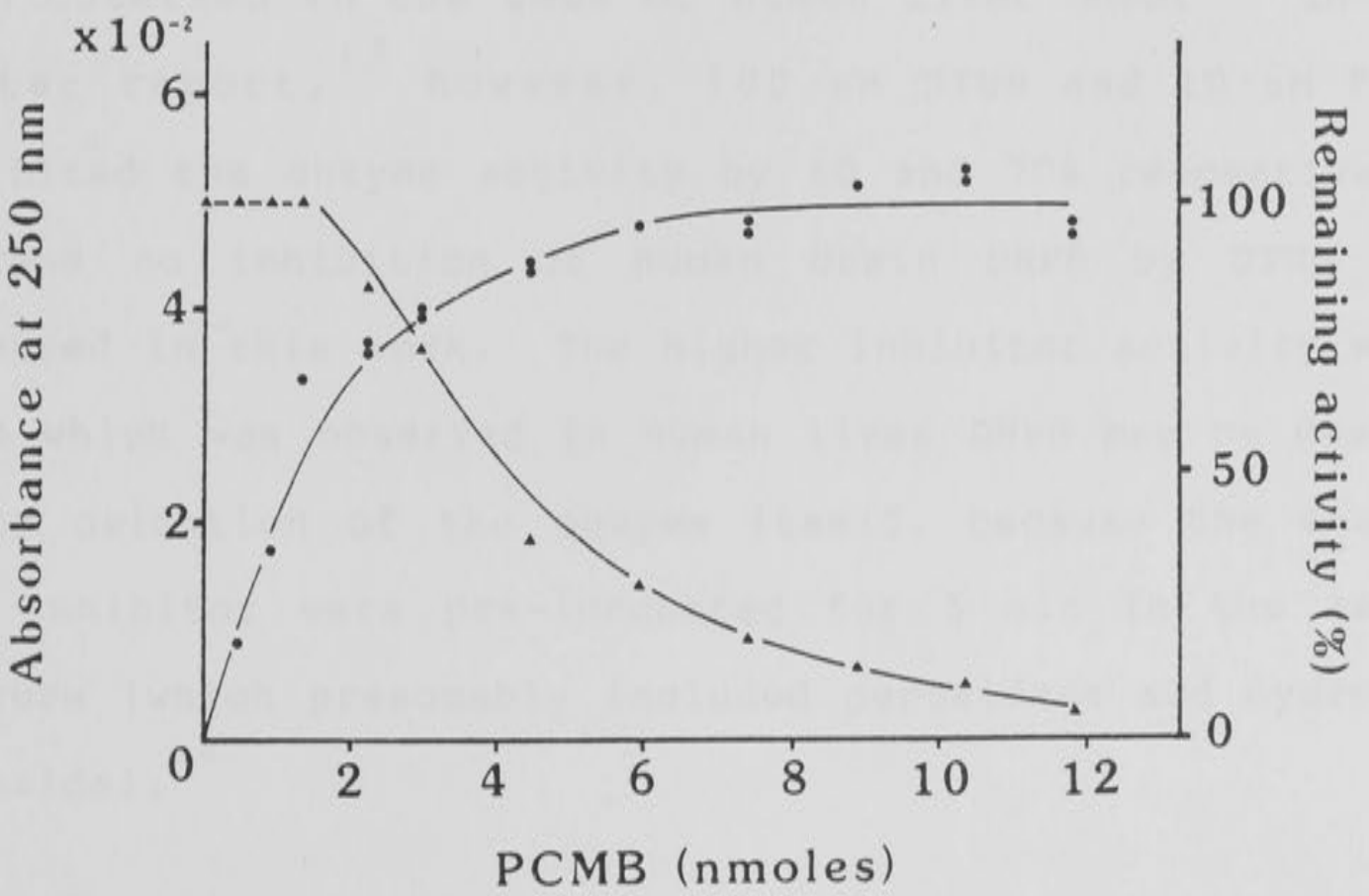


Figure 11. Titration of DHPR with PCMB (pH 7.0) at 25°C.

-●- : DHPR (DTT free, 50 µl, 840 pmol) in 50 mM potassium phosphate buffer (pH 7.0) (250 ml) was titrated with 74.5 µM PCMB (pH 7.0). The blank cuvette contained 0.1 M phosphate buffer (pH 7.2) (50 µl) and 50 mM phosphate buffer (pH 7.0) (250 µl). -▲- : The enzyme activity was monitored in duplicate. 3 µl of enzyme samples were removed after equilibration with each aliquot of PCMB and assayed.

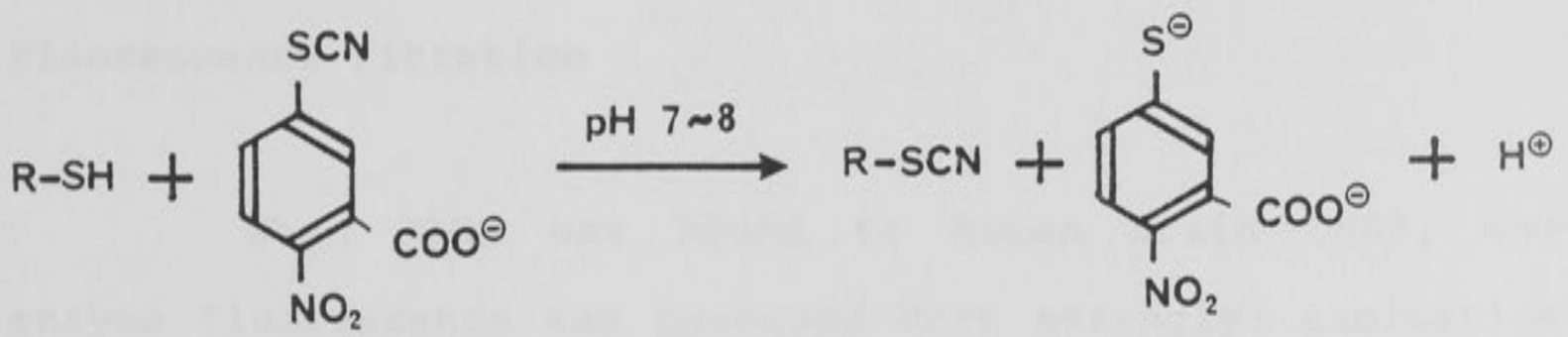
The titration curve at pH 7.0 (Figure 11) was not as sharp as at pH 4.6. The curved portion was at about 3.2 to 3.3 -SH equivalent of PCMB (3 -SH by extrapolation). This curvature is probably due to the slower reactivity of the second and third -SH groups. By assaying the aliquots for enzyme activity during the titration it was found that the activity decreased after the second -SH group was titrated (Figure 11). This is in a good agreement with the result from DTNB estimation. Protection by NADH from the inhibition caused by PCMB was observed when DHPR was pre-incubated with NADH (45.5 μM) (no -SH group reacted with PCMB). This protection by NADH against PCMB inhibition was also observed in the case of human liver DHPR.[10] In the latter report,[10] however, 100 μM DTNB and 10 μM PCMB inhibited the enzyme activity by 60 and 70% respectively, whereas no inhibition of human brain DHPR by DTNB was observed in this work. The higher inhibitor activity with DTNB which was observed in human liver DHPR may be due to prior oxidation of the enzyme itself, because the enzyme and inhibitor were pre-incubated for 5 min in the assay mixture (which presumably included peroxidase and hydrogen peroxide).[44]

2-6-3 Reaction with 2-nitro-5-thiocyanobenzoic acid (NTCB)

The 2-nitro-5-thiocyanobenzoic acid (NTCB) assay was developed to introduce a small and neutral group for reaction with thiol residues in the native protein and for

comparison with other thiol specific reagents.[119] Both DTNB and PCMB are bulky by comparison and it is difficult to ignore the effects from their size on their reactions with proteins. In the case of PCMB, there was still doubt as to whether the inhibition was caused by the reaction between PCMB and the catalytic cysteine residues in the enzyme or the bulkiness of PCMB molecule which bound to non-catalytic cysteine residues close to the active site.

NTCB was incubated in the same way as DTNB.[119] In both non-denatured and denatured conditions the colour development at 412 nm was very poor even with reasonably high NTCB concentrations. Only 0.5 ~ 0.9 -SH per subunit (ca one -SH) was titrated. (All solutions were freed from oxygen by bubbling nitrogen gas.) The proposed reaction is shown in Scheme 3.[119] It was carried out in 0.1 M



Scheme 3

phosphate buffer (pH 7.2), so it was unlikely that the pH of the solution dropped after the release of protons.

Theoretically NTCB should react with most of the -SH groups. The poor affinity of NTCB for the -SH groups of this protein cannot be readily explained. It is possible that some -SH groups are less readily ionized at pH 7.2 and therefore less nucleophilic.

2-7 Stoichiometry of DHPR and NADH

Now, as the concentration of DHPR has been determined accurately, it is possible to obtain good estimations of the stoichiometry of the reaction with NADH. Stoichiometric studies of the binding of NADH to the DHPR molecule from other sources have given two different results; (a) one molecule of NADH binds to one subunit (* the active form of DHPR from all sources has two identical subunits), and (b) one molecule of NADH binds to two subunits. The different results may be due to incorrect protein concentration estimation (see p 43) or may depend on the sources of DHPR.

In this work, the determination of the stoichiometry was carried out by fluoriometric titration.

Fluorescence titration

When NADH was bound to human brain DHPR, the enzyme fluorescence was quenched very strongly; excitation wavelength was 280 nm and emission spectra were scanned from 310 to 550 nm (Figure 4, p 50). Similar behaviour was observed with rat liver DHPR.[82,86] The fluorescence intensity of DHPR at 350 nm decreases concurrently as NADH is added until the binding is complete and then remains steady as more NADH is added. Under the same conditions, free NADH has almost no fluorescence, whereas NADH bound to DHPR causes an increase in the fluorescence at the

wavelength maximum 469 nm. After saturation of the DHPR active site with NADH, the fluorescence at 469 nm does not increase further.

In this work, the reaction between NADH and human brain DHPR was followed by observing the changes in the fluorescence of NADH at 470 nm. The titration curve is depicted in Figure 12.

NADH did not bind to human brain DHPR which was denatured thermally or with 4 M guanidine.HCl. After incubating the enzyme at 37^0C for 24 h and 45^0C for 21 h, this enzyme was titrated with NADH but no fluorescence changes at 350 nm (for protein) and at 470 nm (NADH) were observed (Figure 7, p 54). In this experiment, the temperature used might be too high and may have caused complete unfolding of the protein molecule. In 4 M guanidine.HCl solution, the wavelength maximum of protein fluorescence was shifted to 362 nm due to denaturation and the fluorescence at this wavelength slowly decreased after addition of NADH, although no increase of the fluorescence due to bound NADH was observed at 470 nm (Figure 6, p 53). This decrease may be due to slow denaturation of the enzyme in the weak guanidine.HCl solution.

When human brain DHPR (DTT free) (408 pmoles) was titrated with aliquots of 13.9 µM stock NADH solution in 0.1 M potassium phosphate buffer (pH 7.2), the titration curve indicated that one molecule of NADH was bound to 1.3 molecules of the **active** subunit. (The active enzyme seems to be 50% of the total protein concentration, because

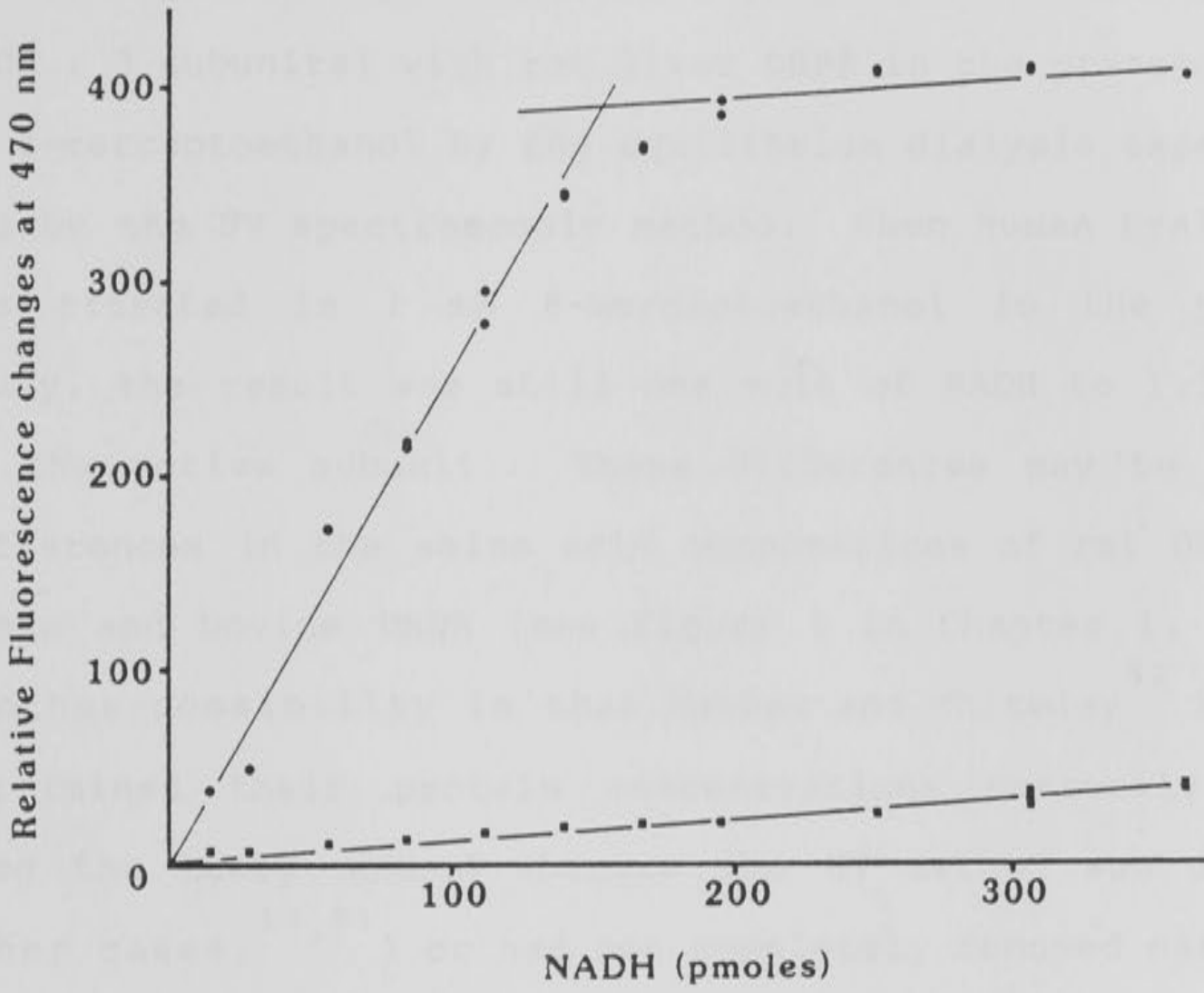


Figure 12. Titration of DHPR with NADH at 23^{0}C.

-●- : DHPR (DTT free, 30 µl, 204 pmol) in 0.1 M phosphate buffer (pH 7.2) (470 µl) was titrated with 13.9 µM NADH at 23^{0}C. -■- : Titration curve of blank contained 0.1 M phosphate buffer (pH 7.2) (500 µl). The excitation and emission wavelengths were 280 nm (slit 5 nm) and 470 nm (slit 10 nm) respectively.

the specific enzyme activity had decreased to half of the original value, whereas the total protein concentration of DHPR did not change.) This result is in a good agreement with the results obtained for bovine liver DHPR.[10,81] Webber and Whiteley[82] reported a different stoichiometry (1 NADH : 2 subunits) with rat liver DHPR in the presence of 1 mM β-mercaptoethanol by the equilibrium dialysis experiment and by the UV spectroscopic method. When human brain DHPR was titrated in 1 mM β-mercaptoethanol in the present study, the result was still one mole of NADH to 1.3 moles of the active subunit. These differences may be due to differences in the amino acid compositions of rat DHPR and human and bovine DHPR (see Figure 1 in Chapter 1, p 24). Another possibility is that Webber and Whiteley[82] had not determined their protein concentrations correctly (they used the Lowry method whereas the UV method was used in other cases,[10,81]) or had not completely removed naturally bound NADH from their protein.

The dissociation constant of the binding of NADH to human brain DHPR was calculated from the titration by observing the changes of the protein fluorescence at 470 nm (Figure 12, p 67).

The Kd value was determined using a computer programme kindly supplied from Dr J.F. Morrison[120] and found to be 13.8 ± 3.2 nM. This corresponded to a total protein concentration of 74.1 ± 1.6 nM. This total enzyme concentration was almost half the value of that obtained from the Bio-Rad estimation (160 nM). However, the enzyme

used in this experiment had lost about half its enzyme activity, so the true active protein concentration was ca 80 nM which was in a good agreement with the value from the computer evaluation. The Kd value for human brain DHPR of 13.8 is to be compared, considering the errors involved, with the Kd values of 78 ± 24 nM[86] and 166 ± 74 nM[82] reported by different authors for rat liver DHPR (see reference 22).

2-8 Conclusion

The Bio-Rad assay and UV method (ε 92,800 $M^{-1}.cm^{-1}$ at 280 nm) were found to be the most reliable methods for estimation of the protein concentration of human brain DHPR The Lowry method gave high protein values.

The amino acid composition of human brain DHPR (Table 1, p 42) was similar, within the error of the method used, to the reported composition for human liver DHPR. Human brain DHPR has 5 or 6 tryptophan residues per subunit and most of those residues are highly exposed to the solvent. Three or four cysteine residues were estimated from the amino acid analysis whereas only three residues were obtained from the reaction with thiol specific reagents. This difference could be due to experimental error but another possibility was that the fourth cysteine residue was located at a highly hydrophobic region of the protein and was not readily accessible to thiol reagents even under the denaturing conditions. Experiments revealed

that human brain DHPR had one free cysteine residue per subunit on the outside of the molecule which did not affect enzyme activity. The second and third cysteine residues may be of significance for enzyme activity, although the inactivation of the enzyme was slow after the first thiol group was reacted (cf. Chapter 4 on DHPR inactivation by platinum(II) complexes).

One mole of NADH cofactor binds to one mole of the DHPR subunit with a Kd value of 13.2 nM.

For the definitive amino acid composition, we have to wait for the results from amino acid sequence studies and from X-ray crystallographic analysis.

2-9 Experimental

2-9-1 General Methods

A: Chemicals

All commercial chemicals were of the highest commercially available purity.

DEAE-Sephadex A-50 was from Pharmacia, Uppsala, Sweden. 6-Methyl-5,6,7,8-tetrahydropterin hydrochloride was supplied by Dr W.L.F. Armarego. Visking dialysis tubing (Scientific Instrument Centre Ltd., London) was stored at 4^0C in 50% ethanol and hydrated before use by soaking overnight in distilled water.

All concentrations in parentheses are the final concentrations.

B: Methods

Polyacrylamide gel electrophoresis[121] was carried out according to the method of Ornstein and Davis[121] (60 x 80 x 1 mm running gel was used). Pharmacia low molecular weight standards (Uppsala, Sweden) were used as molecular weight markers.

The gels were stained with Serva Blau R-250 (Serva, Heidelberg, WG) or with silver staining.[122]

SDS-polyacrylamide gel electrophoresis[121] was carried out according to the method of Laemmli (60 x 80 x 1 mm gel was used).

Amino acid analysis was carried out using a Beckman Systems 6300 HPLC analyser in the Department of Biochemistry by Mr L.B. James.

A Perkin-Elmer Lambda 1 single beam UV spectrometer was used for the determination of protein concentrations and the thiol group estimation with DTNB or NTCB. Ultraviolet spectra and kinetic assays were performed on a double beam Unicam SP 1800 connected to a Rikadenki B-281 H recorder with 5 mV across the slide wire to produce a maximum pen movement corresponding to 0.1 absorbance units. For higher sensitivity a Cary 219 spectrometer adjusted to produce maximum pen movement for 0.01 absorbance units was used. The cell holders were thermostated at the desired temperatures with re-circulating water from a Coolnics thermostat unit (model CTE-18, Komatsu-Yamato, Japan).

Fluorescence spectra were measured with a Perkin-Elmer LS-5 Luminescence spectrometer using 280 nm as excitation wavelength (slit 5 nm) and the fluorescence emission was measured at 350 or 470 nm (slit 10 nm) or was scanned (slit 5 nm). A 4 mm thermostated quartz cuvette was used at the desired temperature and the excitation beam was given only when fluorescence readings were noted.

The assays were performed as described by Nielsen[9] except that potassium ferricyanide was used as oxidant.[49] The experiment and blank cuvettes had the same ingredients except for the experimental cuvette into which the enzyme solution was injected. Both cuvettes contained

distilled water (805 µl; or 755 to 785 µl for enzyme purification) and 1 M Tris/HCl buffer (pH 7.4) (100 µl, 0.1 M), and were kept at 25^{0}C prior to the assay. The enzyme solution (1 µl; or 20 to 50 µl for enzyme purification) was added to the experimental cuvette and then 6-MeTHP (20 µl dissolved in 4 mM HCl, 30.2 µM), and NADH (25 µl dissolved in 50 mM Tris/HCl buffer, pH 7.3, 119.1 µM) were added to both cuvettes simultaneously. The reaction was initiated by addition of 2 mM potassium ferricyanide (50 µl, 100 µM). The oxidation of NADH to NAD^+ was observed at 340 nm, 25^{0}C, and the rate of reaction was obtained from the concentration of oxidized NADH (absorbance change at 340 nm) per minute by using 6.2 x 10^3 $M^{-1}.cm^{-1}$ as the extinction coefficient of NADH.

Kinetic assays were run in duplicate.

Small volumes of enzyme used in kinetic runs were dispensed with a Hamilton Whittier-cal PB 600 dispenser and a 50 µl Hamilton syringe which can deliver 1 µl at a time.

2-9-2 Enzyme purification

The active fractions from the naphthoquinone affinity chromatography (see Section 2-2, p 38) were collected and dialyzed against 50 mM Tris/HCl buffer (pH 7.4) for 24 h at 4^{0}C. This dialysate was concentrated to small volumes (10 ~ 15 ml) in an Amicon PM-10 membrane filter (Amicon Co., Danvers, USA.), and applied to a DEAE-Sephadex A-50 column (2 x 35 cm) with 50 mM Tris/HCl buffer

(pH 7.8). The column was eluted with a gradient of 0 to 250 mM KCl in the same buffer at 4^0C. The protein concentrations of the fractions were measured by UV absorption (at 280 nm) and by the Bio-Rad micro assay. The enzyme activity was also measured for these fractions. The active fractions eluted at about 100 ~ 150 mM KCl. These were collected and 1 ml portions were stored in glass vials at -20^0C (these were designated as stock DHPR). When required, these solutions were thawed and dialyzed against the above buffer or the buffer which contained 2 mM DTT at 4^0C. Some protein precipitated on dialysis and was removed. Because the enzyme gave the same band (one band) on SDS-PAGE before and after dialysis it appeared that the protein that had precipitated on dialysis was inactivated DHPR (see Table 4).

2-9-3 Amino acid analysis[106]

A: The protein was alkylated with iodoacetic acid prior to amino acid analysis. Dialyzed DHPR solution (DTT free, 100 µl) in 0.1 M potassium phosphate buffer was lyophilized in screw-cap glass vials. The residue was re-dissolved in buffer solution (50 µl; 6 M guanidine.HCl, 0.1 M Tris and 1 mM EDTA solution, pH 8.3) and 3.9 mM β-mercaptoethanol (129 µl) was added. The air in the vials was displaced by blowing nitrogen gas through them and the solution was incubated for *ca* 10 h at 37^0C. Iodoacetic acid (recrystallized from chloroform and then petroleum ether)

Table 4 Enzyme purification on DEAE-Sephadex A-50

	Total protein[a] (mg)	Total activity[b] (u)
DEAE-Sephadex A-50		
stock DHPR	12.2	1928
dialyzed DHPR	8.4	1932

a) Protein concentration was obtained by the Bio-Rad microassay.

b) unit (μmoles NADH oxidized per min)

in water (50 mM, neutralized with NaOH) (12 µl) was added to the previous solution and again flushed with nitrogen gas and capped. The mixture was incubated at room temperature for 30 min. This reaction mixture was passed through a Sep-pak column (Waters Associates, Ma., USA.; which was pre-equilibrated with 80% acetonitrile in 0.1% aqueous trifluoroacetic acid and then with 0.1% aqueous trifluoroacetic acid), to remove non-protein materials, and the column was washed with 0.1% aqueous trifluoroacetic acid (2 ml) and then with 20% acetonitrile in 0.1% aqueous trifluoroacetic acid (2 ml). Protein was eluted from the column with 80% acetonitrile in 0.1% aqueous trifluoroacetic acid (4 ml) and the eluate was evaporated *in vacuo* over KOH pellets. This protein residue was hydrolyzed with 6 M HCl at 110^0C for 22 h and the amino acid composition was determined.

B: The performic acid oxidation of protein was performed as follows. The buffer in the protein solution (50 µl) was removed by passing the solution through the Sep-pak column (see above) and dried. Performic acid [1 ml, prepared from 90% formic acid (9.5 ml) and 30% hydrogen peroxide (0.5 ml)] was added to the protein residue in an ice-bath. The oxidation was allowed to proceed at 0^0C for 6.5 h and the solution was evaporated to dryness. The dried (*in vacuo* over KOH) residue was hydrolyzed with 6 M HCl at 110^0C for 22 h in a sealed tube. Bovine β-lactoglobulin B (Mr 18,400) was used as the standard protein. It was dissolved

in the same buffer as used for DHPR and then treated as above for DHPR.

The estimated error for pipetting was obtained by weighing and was ± 1.1%. This value was applied for the calculation of total protein concentration.

2-9-4 **Spectrophotometric determination of the numbers of tryptophan and tyrosine residues**

Solution A: 8 M guanidine.hydrochloride in 25 mM phosphate buffer (pH 6.33).

Solution B: 8 M guanidine.hydrochloride in 0.13 M NaOH (pH 11.8).

The method of Goodwin and Morton[108] made use of the UV absorptions of tyrosine and tryptophan in alkaline (295 nm) and neutral (280 nm) pH. The experiment was carried out using a single beam UV spectrometer at 25^{0}C. The dialyzed DHPR solution (containg 2 mM DTT, 50 µl, 92 pmol) in 50 mM potassium phosphate buffer (pH 7.2) was mixed with solution A (150 µl, final concentration of guanidine.HCl was 6 M) in a cuvette and the optical density was read at 280, 288 and 295 nm. The corresponding blank experiment contained 50 mM phosphate buffer (50 µl) instead of enzyme solution. The optical density of DHPR in alkaline pH was read at 295 nm after mixing the dialyzed DHPR solution (2 mM DTT, 50 µl) and solution B (150 µl). The blank value was measured by using the corresponding buffer solution.

If the absorbance of the protein in alkaline and neutral pH can be measured then an estimate of the number of tryptophan residues can be made from a knowledge of the number of tyrosine residues (obtained from equation 2 and general amino acid analysis). According to Goodwin and Morton's method,[108] the ratio can be obtained from the following equation (3);

$$A_{280.neutral} = 5690 \times N_{trp} + 1280 \times N_{tyr} \quad \text{--- (1)}$$

$$A_{295.alkaline} = 2480 \times N_{tyr} + A_{295.neutral} \quad \text{--- (2)}$$

$$N_{trp}/N_{tyr} = \frac{A_{280.neut} - 0.516(A_{295.alk} - A_{295.neut})}{A_{295.alk} - A_{295.neut}} \times 0.44 \quad \text{--- (3)}$$

where A is absorbance observed, N is number of amino acids, 5690 is the extinction coefficient of tryptophan at 280 nm in neutral pH, 1280 is the extinction coefficient of tyrosine at 280 nm in neutral pH, and 2480 is the extinction coefficient of tyrosine at 295 nm in alkaline pH.

On the other hand, Edelhoch proposed the determination of the number of moles of tyrosine and tryptophan per mole of protein in neutral solution by

measuring the UV absorption at 280 and 288 nm. When this method was applied to DHPR, it gave negative values for the ratio, so N_{trp} was obtained from the neutral pH values which were derived from equation (4), and N_{tyr} was obtained from equation (2) and the ratio from equation (5).

$$N_{trp} = (0.322 \times A_{288.neut} - 0.0969 \times A_{280.neut}) \times 10^{-3} \quad --(4)$$

$$N_{trp}/N_{tyr} = \frac{(0.322 \times A_{288.neut} - 0.0969 \times A_{280.neut})}{0.4032(A_{295.alk} - A_{295.neut})} \quad ---(5)$$

The results obtained were applied to equations (3) and (5), and both results should give the same value within experimental error (see Table 5).

2-9-5 The Bio-Rad micro assay

DHPR solution (DTT free, 10µl) in 0.1 M potassium phosphate buffer (pH 7.2) was dissolved into water (790 µl) and the Bio-Rad reagent (200 µl) was added to it, mixed well, and then set aside at room temperature for 10 min. The optical density was measured at 595 nm. The corresponding buffer solution (50 µl) was used as blank. The protein concentration was evaluated from the standard curve using BSA. The determinations were carried out in duplicate.

Table 5. The ratio of N_{trp} to N_{tyr}.

	A_{neut}			A_{alk}	N_{trp}/N_{tyr}		
	280	288	295 nm	295 nm	a	b	c
DHPR human brain (dialyzed)	0.171	0.156	0.114	0.155	1.6	2.0	——
β-lacto-globulin	0.177	0.140	0.081	——	–	0.5	0.5[d]
DHPR human liver	——	——	——	——	–	–	1.67[e]

a : calculated from equation (3)

b : calculated from equation (5)

c : calculated from known amino acid composition

d : cf. reference 107

e : cf. reference 10

2-9-6 Lowry method

Solution A : Na_2CO_3 (20 g) + NaOH (4 g) /L of H_2O

Solution B : Sodium tartrate (1 g) was dissolved in water (100 ml) and then $CuSO_4.5H_2O$ (0.44 g) was added to it. The supernatant was used.

Solution C : Prior to use, solution A and solution B were mixed in the ratio of 50 : 1.

Folin solution : Folin-Ciocalteau's phenol reagent (BDH, Poole, England) : H_2O = 2 : 3

DHPR solution (DTT free or 2 mM DTT, 20 µl) was dissolved in H_2O (180 µl) and solution C (1 ml) was added to it and then left at room temperature for 10 min. The Folin solution (0.5 ml) was added and after 30 min the absorbance at 650 nm was measured. The blank was carried out with H_2O. The protein concentration was deduced from a curve using BSA as a standard protein.

2-9-7 The fluorescence spectra of DHPR

A: Non-denatured DHPR

i) DHPR (DTT free) (10 µl, 0.14 µM) was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) (490 µl) and the emission spectra were scanned at 20^0C (Figure 3, p 48).

ii) DHPR (DTT free) (10 µl, 0.14 µM) was mixed with NADH (10 µl, 0.8 µM) in 0.1 M potassium phosphate buffer (pH

7.2) (490 µl). The fluorescence emission spectra were scanned at 20^{0}C (Figure 4, p 50).

iii) DHPR (DTT free) (10 µl, 0.14 µM) was mixed with 6-MeTHP (10 µl in 4 mM HCl, 43 µM) in 0.1 M potassium phosphate buffer (pH 7.2) (490 µl), and potassium ferricyanide (10 µl, 100 µM) was added to it. The emission spectra were scanned at 20^{0}C (Figure 5, p 51).

B: Denatured DHPR

i) DHPR (DTT free) (10 µl, 0.14 µM) was denatured in 8 M guanidine.HCl in 0.1 M potassium phosphate buffer (pH 7.0) (490 µl) and the emission spectra were scanned at 20^{0}C. Although the fluorescence maximum of DHPR shifted to 374 nm, this 8 M guanidine.HCl in buffer itself had a maximum at 384 nm and its intensity was 78% of that of DHPR at the same wavelength. This bathochromic shift of enzyme fluorescence seems to be due to guanidine.HCl.

ii) DHPR (DTT free) (5 µl, 0.07 µM) was denatured in 4 M guanidine.HCl in 0.1 M phosphate buffer (pH 7.0) (495 µl), and then NADH (10 µl, 0.8 µM) was added. The fluorescence emission spectra showed no quenching due to NADH-(denatured DHPR) binding (Figure 6, p 53).

iii) Thermally denatured DHPR (at 37^{0}C for 24 h and 45^{0}C for 21 h; 10 µl, 0.14 µM) was dissolved in 0.1 M phosphate buffer (pH 7.2) (490 µl) and then incubated with NADH (10

µl, 0.8 µM). The fluorescence spectra showed no quenching based on NADH binding to DHPR (Figure 7, p 54).

iv) DHPR (DTT free) (5 µl) was denatured in 4 M urea in 0.1 M phosphate buffer (pH 7.6) (595 µl) at 20^{0}C and the fluorescence spectra were scanned. The emission maximum shifted with time to longer wavelength and with decreasing fluorescence (Figure 8, p 55).

2-9-8 Cysteine estimation

A: DTNB

DTNB solution for the estimation of -SH groups in non-denatured enzyme was made from DTNB (40.68 mg) in 0.1 M potassium phosphate buffer (pH 7.0, 10 ml). Dialyzed DHPR (DTT free) (150 µl, 9.96 µM) was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) (100 µl) and the above 10 mM DTNB solution (pH 6.8) (30 µl, final concentration 1.07 mM) was added and then this mixture was incubated at 21^{0}C. The absorbance changes at 412 nm were measured with a single beam spectrometer and the reaction was complete after 4.5 h. Blank incubations were carried out with 0.1 M potassium phosphate buffer (pH 7.2) instead of DHPR solution. The change of absorbance due to the protein-DTNB reaction was 0.135 O.D. and this was calculated as 1.0 cysteine residue by using 13,600 $M^{-1}.cm^{-1}$ as the extinction coefficient for the released TNB anion.

Concurrently, the enzyme activity was measured at

intervals of time by assaying aliquots (1 µl) from the incubation cuvette. No change in enzyme activity was observed during the first 5.3 h of incubation.

The estimation of total -SH groups in DHPR was carried out by incubating DHPR (DTT free, 50 µl, 4.2 µM) with 10.3 mM DTNB (pH 7.0) (5 µl, final concentration 0.24 mM) in 8 M guanidine.HCl in 0.1 M phosphate buffer (pH 7.0) (150 µl) at 21^0C. The blank experiment was carried out with 0.1 M potassium phosphate buffer (pH 7.2) (50µl) instead of enzyme solution. The absorbance at 412 nm started to decrease soon after mixing, because of the released TNB anion. Under the same conditions, 10.3 mM DTNB (pH 7.0) (200 µl, final concentration 5 mM) was incubated with DHPR (DTT free, 50 µl, 2.2 µM) in the above buffer (pH 7.0) (150 µl) and this revealed the presence of 3.5 cysteine residues per DHPR subunit.

DHPR (DTT free, 75 µl, 5.1 µM) was pre-incubated with 4 mM NADH in 50 mM phosphate buffer (pH 7.2) (10 µl, 140 µM) and 0.1 M potassiumm phosphate buffer (pH 7.2) (180 µl) for 5 min at 21^0C and then mixed with 10 mM DTNB (pH 6.8) (15 µl, final concentration 536 µM). This was incubated for 4.4 h and gave a value of 1.4 cysteine residues per subunit. The enzyme activity was examined from time to time by taking aliquots (1 µl) from the incubation cuvette and ca 34% enhancement of DHPR activity was observed (no time dependent loss of enzyme activity was noticed). The blank incubation was carried out by using 0.1 M potassium phosphate buffer (pH 7.2) (255 µl) and the

corresponding NADH solution (10 µl).

B: PCMB

Stock solution : PCMB (9.1 mg) and 0.04 M NaOH (1 ml) were diluted to 25 ml with water and this was kept in dark at room temperature.

Solution A : The above stock solution (2.0 ml) was diluted to 25 ml with 50 mM potassium phosphate buffer (pH 7.0). The solution was centrifuged prior to use to remove any precipitate formed.

Solution B : The stock solution (2.0 ml) was diluted to 25 ml with 0.33 M sodium acetate buffer (pH 4.6). The solution was centrifuged prior to use to remove any precipitate formed.

i) Standardization with glutathione

Glutathione stock solution was freshly prepared by dissolving glutathione (20.3 mg) in water (10 ml). PCMB solution (1 ml of the solution A or B) was placed in the cuvette and corresponding buffer (1.0 ml) in the blank cuvette. The titration was carried out by delivering stock glutathione solution to both cuvettes and mixing with a glass rod. The absorption was measured at 250 nm for the pH 7.0 buffer or at 255 nm for the pH 4.6 buffer. The observed absorbances were corrected for dilution and plotted against the concentration of glutathione added.

ii) Determination of total thiol groups

DHPR (DTT free, 50 µl, 840 pmol) in 0.33 M sodium acetate buffer (pH 4.60) (250 µl) was placed in one cuvette with 0.1 M potassium phosphate buffer (pH 7.2) (50 µl) in 0.33 M sodium acetate buffer (pH 4.60) (250 µl) in the blank cuvette at 25^{0}C. These two cuvettes were titrated with solution B, 36.3 µM PCMB, (10 µl aliquots) and the changes at 255 nm were observed. This estimated 2.8 -SH groups per enzyme subunit. Moreover, the same experiment was carried out in the 0.33 M sodium acetate buffer (pH 4.6) which contained 2% of SDS. In this case 2.9 -SH groups were estimated per DHPR subunit.

iii) Determination of active thiol groups

DHPR (DTT free, 50 µl, 840 pmol) in 50 mM potassium phosphate buffer (pH 7.0) (250 µl) was placed in a cuvette, and 0.1 M potassium phosphate buffer (pH 7.2) (50 µl) in 50 mM phosphate buffer (pH 7.0) (250 µl) was in the blank cuvette at 25^{0}C. The titration was carried out with solution A, 74.5 µM PCMB, (3 µl aliquots) (Figure 11, p 62). This revealed ca 3.2 to 3.3 -SH per enzyme subunit. The enzyme activity was assayed at the same time by taking aliquots (3 µl) of solution from the cuvettes; the activity decreased after the second -SH group was titrated. The enzyme activity was not restored after dialyzing this incubation solution against 50 mM phosphate buffer (200 ml x 3) for 18 h at 4^{0}C.

When DHPR (DTT free, 50 µl, 3.2 µM), NADH (100 µl,

45.5 µM) and 50 mM phosphate buffer (pH 7.0) (150 µl) were placed in the experimental cuvette, and 0.1 M phosphate buffer (pH 7.2) (50 µl), NADH (100 µl, 45.5 µM) and 50 mM phosphate buffer (pH 7.0) (150 µl) were in the blank cuvette, and those were titrated with solution A (using 4 µl aliquots), no PCMB reacted with DHPR. This absence of colour change might be due to the low protein concentration (3.2 µM). Because in the case of DTNB one -SH was titrated under NADH protection, so also in the case of PCMB reaction, at least one -SH should be titrated. The enzyme activity was unaltered at the end of the PCMB titration.

C: NTCB

The buffer and reagent solutions were flushed with nitrogen gas prior to use.

i) Non-denatured enzyme

DHPR (DTT free, 50 µl, 840 pmol) was incubated with 10 mM NTCB solution (in 0.1 M phosphate buffer, pH 7.2) (100 µl) and 0.1 M phosphate buffer (pH 7.2) (150 µl). The blank cuvette contained 10 mM NTCB solution (100 µl) and 0.1 M phosophate buffer (pH 7.2) (200 µl). The absorbance changes at 412 nm were measured with a single beam spectrometer at 23^0C. The total absorbance change was 0.038 O.D. which indicated 0.9 -SH per subunit when using 13,600 $M^{-1}.cm^{-1}$ as the extinction coefficient for the TNB anion.

When the incubation was carried out with DHPR

(DTT free, 200 µl, 6.6 µM), NTCB (40 µl, 1.4 mM) and 0.1 M potassium phosphate buffer (pH 7.2) (50 µl), ca 0.5 -SH per subunit was estimated. The enzyme activity was measured at the same time, but no inactivation by NTCB was observed.

ii) Denatured enzyme

DHPR (DTT free, 25 µl, 0.96 µM) was incubated with 10 mM NTCB solution (25 µl) and 8 M guanidine.HCl (in 1.3 mM EDTA and 0.1 M phosphate buffer, pH 7.2; 200 µl) and the blank experiment was carried out with the corresponding phosphate buffer in place of enzyme. This experiment also revealed ca one -SH per subunit but the enzyme was still active (80%). DHPR was only partly denatured under these conditions.

2-9-9 Stoichiometry of NADH and DHPR

A: Fluorescence titration

i) DHPR (DTT free, 30 µl, 204 pmoles of active subunit) was diluted with 0.1 M potassium phosphate buffer (pH 7.2) (470 µl) and titrated with 13.9 µM NADH (2 µl aliquots) at 23^0C. The changes [excitation at 280 nm (slit 5 nm)] were observed at 470 nm (slit 10 nm). The blank titration was carried out using 0.1 M phosphate buffer (pH 7.2) (500 µl) instead of the DHPR solution. The changes in fluorescence were plotted against NADH concentration. At the sharp change in slope 155.7 pmoles of NADH were consumed. From

this result, the stoichiometry of the DHPR subunit and NADH was **1.3 : 1**, i.e. **~1 : 1**.

ii) DHPR (DTT free, 10 µl, 68 pmol of active subunit) was diluted with 0.1 M potassium phosphate buffer (pH 7.2) (490 µl) and mixed with ß-mercaptoethanol (35 µl, 1 mM) and then titrated with 40 µM NADH (1 µl aliquots) at 20^{0}C. The relative fluorescence changes were observed at 470 nm (slit 10 nm) with excitation wavelength at 280 nm (slit 5 nm). The blank was carried out with corresponding buffer. At the sharp change in slope, 54 pmoles of NADH were consumed. From this, the stoichiometry of DHPR subunit and NADH was **1.3 : 1**.

B: Dissociation constant of the DHPR - NADH complex

DHPR (DTT free, 10 µl, 80 pmoles) was diluted with 0.1 M potassium phosphate buffer (pH 7.2) (500 µl) and titrated with 48 µM NADH at 23^{0}C. The fluorescence (excitation at 280 nm, slit 5 nm) was observed at 470 nm (slit 10 nm). The observed fluorescence changes at the various volumes of added NADH solutions were computed using a programme which was kindly supplied by Dr J.F. Morrison. The calculated Kd value obtained was 13.8 ± 3.2 nM. From this Kd value the protein concentration was re-calculated as 74.1 ± 1.6 nM by using the following equation;

$$E_t = \frac{L_t + \phi(L_t - Kd)}{\phi(\phi + 1)}$$

where E_t is total protein concentration, L_t is total ligand concentration, Kd is dissociation constant and $\phi = (F_0 - F)/(F_0 - F_\infty)$ (cf. reference 120). Where F_0 is the fluorescence of the free NADH, F is observed fluorescence and F_∞ is the fluorescence of the enzyme-NADH complex.

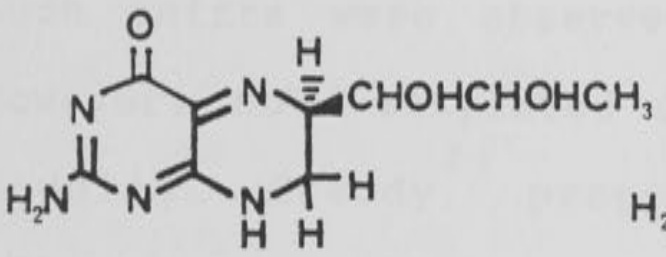
The protein concentration which was estimated from the Bio-Rad microassay was 160 nM, but at the time of the present experiment the enzyme activity had decreased by ca 50%. From a previous experiment [2-9-9 (A), p 88], it is known that NADH can bind only to the active DHPR and produce a change in fluorescence. Thus the real active enzyme concentration is ca 80 nM (160/2 nM) which is in good agreement with the value obtained from the above estimation.

CHAPTER 3

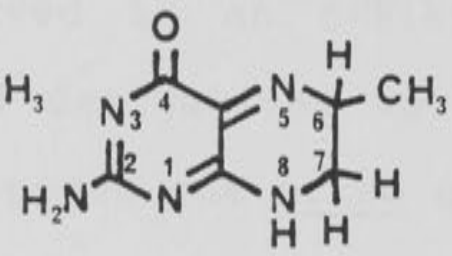
NEW PTERIDINE SUBSTRATES FOR DIHYDROPTERIDINE REDUCTASE

3-1 Introduction

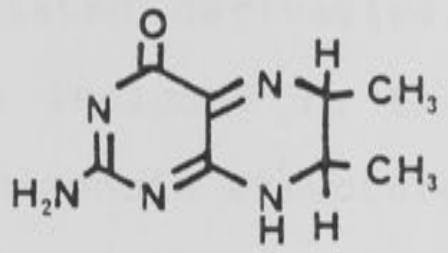
The natural cofactor of dihydropteridine reductase, quinonoid L-erythro-7,8-dihydro(6H)biopterin [1], can be replaced by simple alkylpterins, e.g. quinonoid 6-methyl-7,8-dihydro(6H)pterin [2] and quinonoid 6,7-dimethyl-7,8-dihydro(6H)pterin [3] and still exhibit enzyme activity.[22] These synthetic cofactors are formed



[1]



[2]

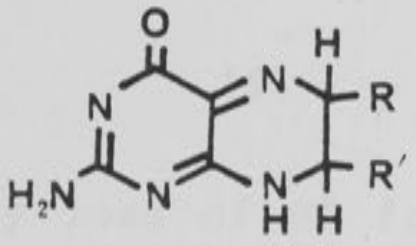


[3]

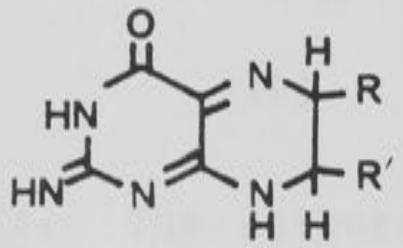
quantitatively from the respective 5,6,7,8-tetrahydropterins at physiological pH by oxidation. The most commonly used method for generating quinonoid dihydropterins for assaying dihydropteridine reductase is that of Nielsen et al.[9] which involves oxidation with peroxidase and hydrogen peroxide.

The structures of the quinonoid dihydropterins produced by the different oxidants and by the aromatic amino acid hydroxylases are the same,[73] and have been the subject of several studies. Quinonoid dihydropterins can exist in three tautomeric forms [4], [5] and [6] in aqueous solution at near neutral pH. Kaufman[68] proposed the para-quinonoid structure [5] after a suggestion by Hemmerich,[123] and presented good evidence for the structure of the

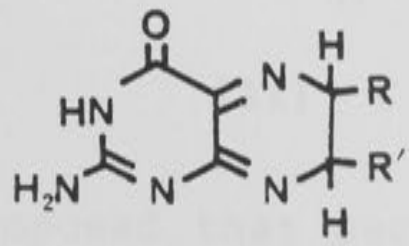
pyrazine moiety [5]. He reported that quinonoid 2-alkylamino-6,7-dimethyl-7,8-dihydro(6H)pterins exhibited



[4]

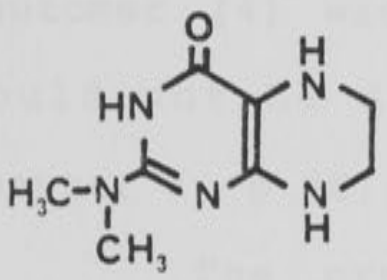


[5]

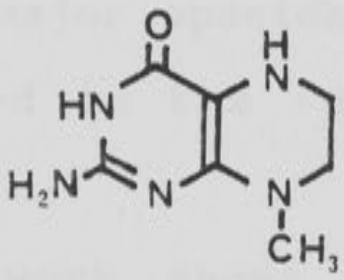


[6]

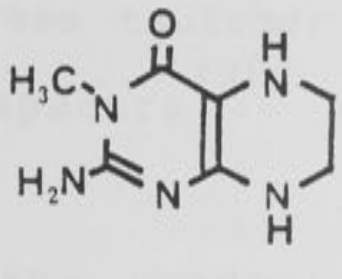
pronounced bathochromic shifts of 15 ~ 20 nm, whereas no such shifts were observed in an 8-alkylated derivative. However, no 3-alkylated derivatives were included in the studies. Gready[71] proposed the ortho quinonoid structure [6] as a possible active substrate by comparing the energies of each tautomer using theoretical methods. The kinetic evidence showed that the tautomer with the endocyclic double bond, i.e. tautomer [4] was the reactive tautomer. For example, Armarego and Waring[72] found that the rates of aerobic oxidation of compounds [7] and [8]



[7]

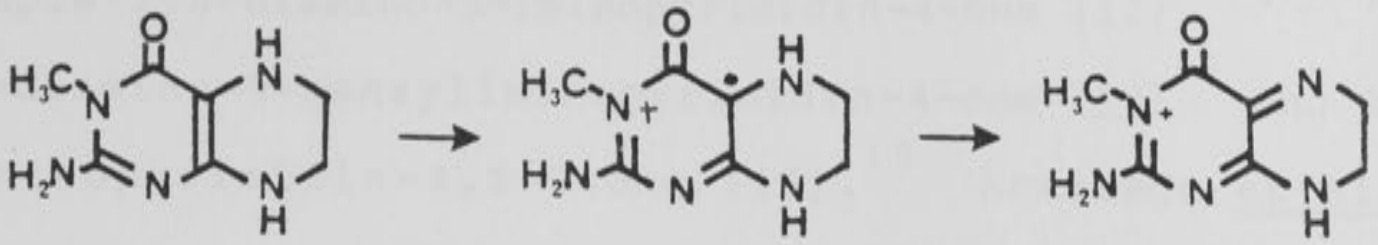


[8]



[9]

were faster compared with compound [9], because of stabilization of the initial radical cation intermediate [10] leading to cation [11]. Such stabilization does not occur in the 3-unsubstituted pterins [7] and [8] because the radical cation would be deprotonated at the neutral pH



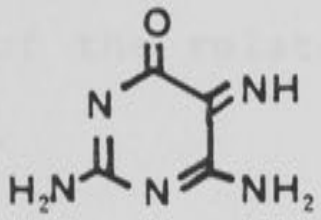
[9] [10] [11]

by loss of H^+ from N3. The authors proposed that such a mechanism should produce the quinonoid species with the ortho structure [4] and not [5] or [6]. In the 3-methyl compound [9], a positive charge will be generated at N-3 to give the quinonoid pterin [11] which should rearrange to the corresponding 7,8-dihydro(3H)pterins much faster than the corresponding quinonoid pterins derived from the other pterins [7] and [8]. This was observed experimentally.[72] ^{1}H NMR studies[73] also excluded the presence of structure [6], although the smaller amounts (~30%) of exocyclic tautomer [5] could not be excluded in the tautomeric equilibrium (see Chapter 1, p 19). Benkovic et al.[74] later used ^{15}N NMR spectroscopy to demonstrate clearly that tautomer [4] was the major species, whereas tautomer [6] could not be detected in the ^{1}H NMR spectra[73(b)] (see Chapter 1, p 20).

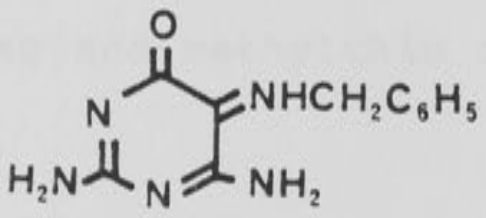
The present work shows that the predominant tautomer [4] in neutral aqueous medium is the enzyme active tautomer. For clarity the quinonoid pteridine structures in this chapter have been written in this tautomeric form, e.g. [1], [2] and [3].

Apart from the pterins, several oxidized pyrimidines have been reported as substrates of DHPR, for

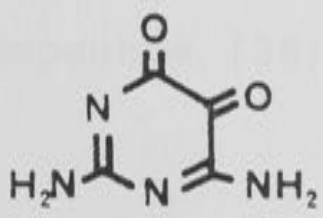
example 2,6-diamino-5-iminopyrimidin-4-one [12],[47,124,125] 2,6-diamino-5-benzyliminopyrimidin-4-one [13][10] and 2,6-diaminopyrimidin-4,5-dione [14].[10] Armarego et al.[47]



[12]



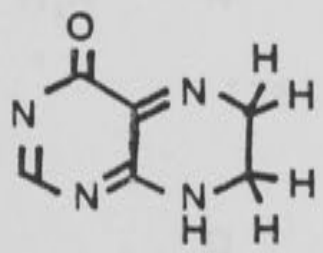
[13]



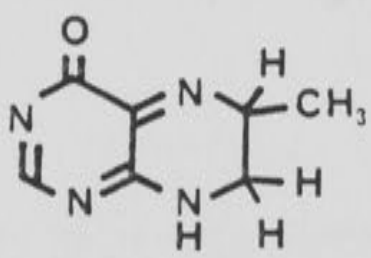
[14]

concluded that the ethylene bridge joining N-5 to N-8 in the normal pterin substrate is not absolutely necessary for substrate activity, but the saturated bridge between the two nitrogen atoms in pterins does aid binding to the active site of the enzyme.

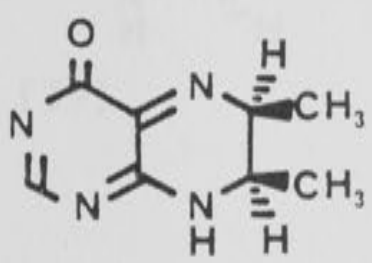
However, the predominant tautomer in solution is not necessarily the structure that is enzymically active. It was therefore argued that if the quinonoid substrate has the tautomeric structure [4], then the quinonoid dihydropteridines [15] to [17], derived from the respective 5,6,7,8-tetrahydropteridinone derivatives, which do not have the 2-amino group, should also be substrates for DHPR.



[15]

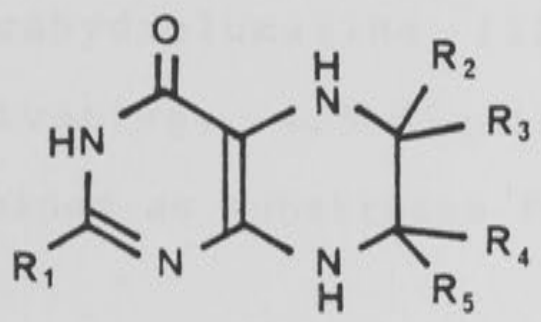


[16]

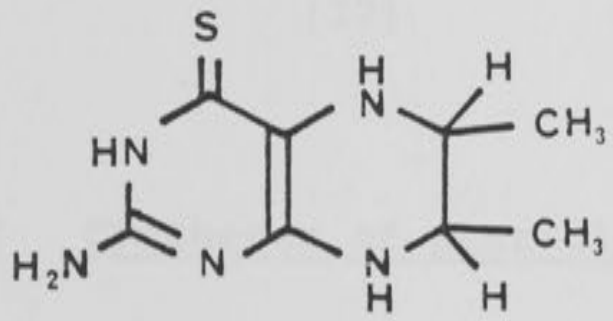


[17]

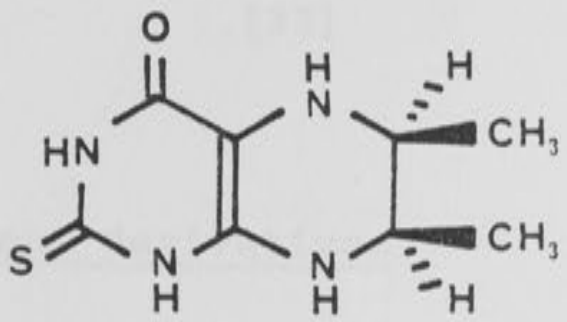
To deduce the tautomeric structure of the substrates at the active site, a study was made of the substrate activities of the quinonoid dihydro compounds obtained by oxidation of the 5,6,7,8-tetrahydropteridin-4(3H)-ones [18] to [25], and of the related thioxo and methylthio compounds [26] to [29].



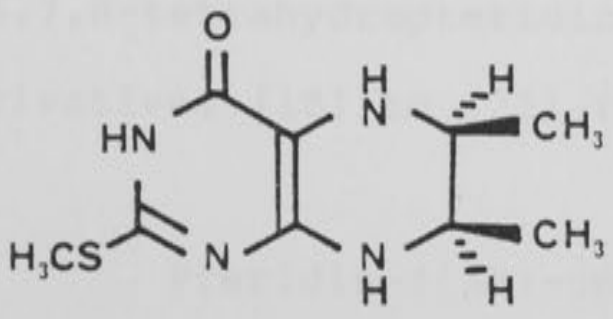
	R_1	R_2	R_3	R_4	R_5
[18]	H	H	H	H	H
[19]	H	D	D	D	D
[20]	H	Me	H	H	H
[21]	H	Me	D	H	D
[22]	H	Me	H	Me	H
[23]	H	Me	D	Me	D
[24]	Me	Me	H	Me	H
[25]	MeNH	Me	H	Me	H



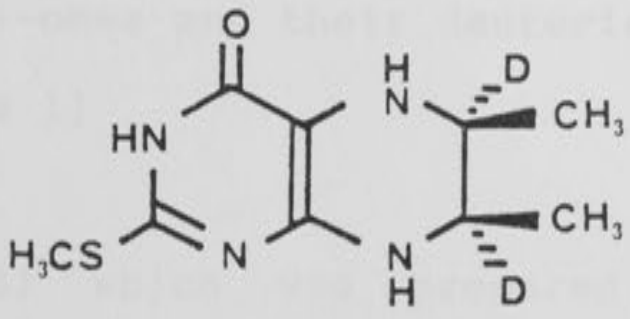
[26]



[27]

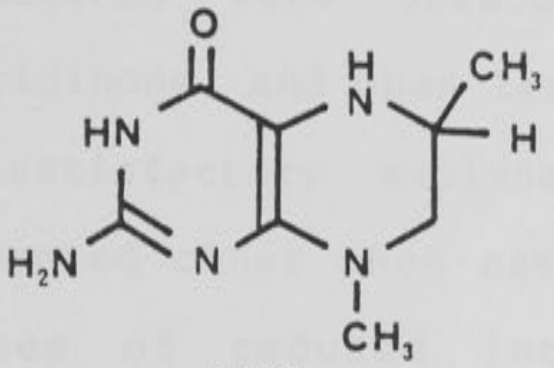


[28]

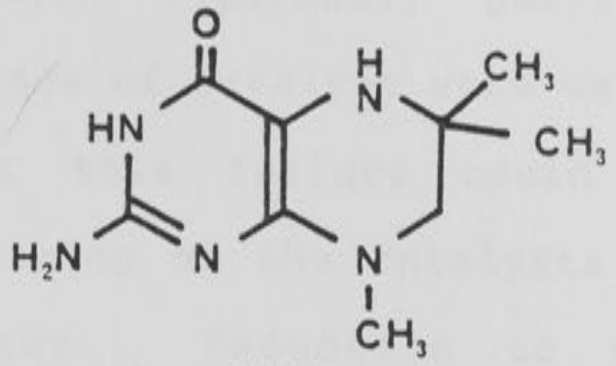


[29]

Most of the tetrahydro compounds used have been synthesized for this work. Also, the quinonoid oxidation products of 6,8-dimethyl-5,6,7,8-tetrahydropterin [30], 6,6,8-trimethyl-5,6,7,8-tetrahydropterin [31],[126] 6-methyl-5,6,7,8-

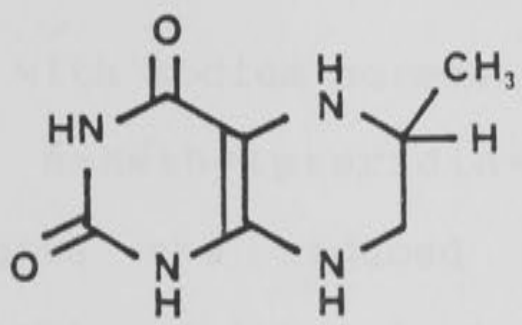


[30]

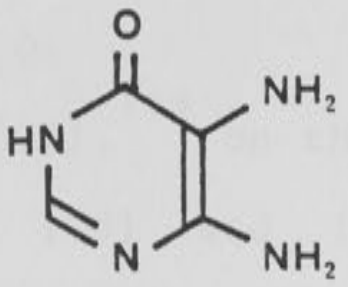


[31]

tetrahydrolumazine [32] and the related pyrimidinone derivative, 4,5-diaminopyrimidin-6(1H)-one [33] were examined as substrates for DHPR.



[32]



[33]

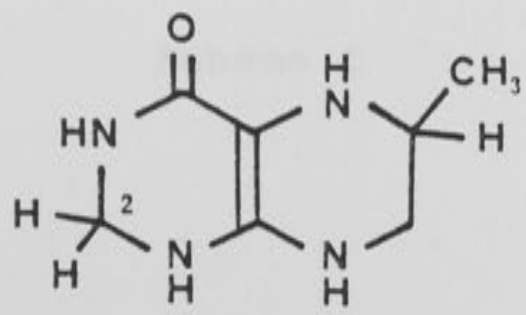
3-2 Syntheses of pteridin-4(3H)-one derivatives

3-2-1 Unsubstituted-, 6-methyl- and cis-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-ones and their deuteriated derivatives [18] to [23] (Scheme 1)

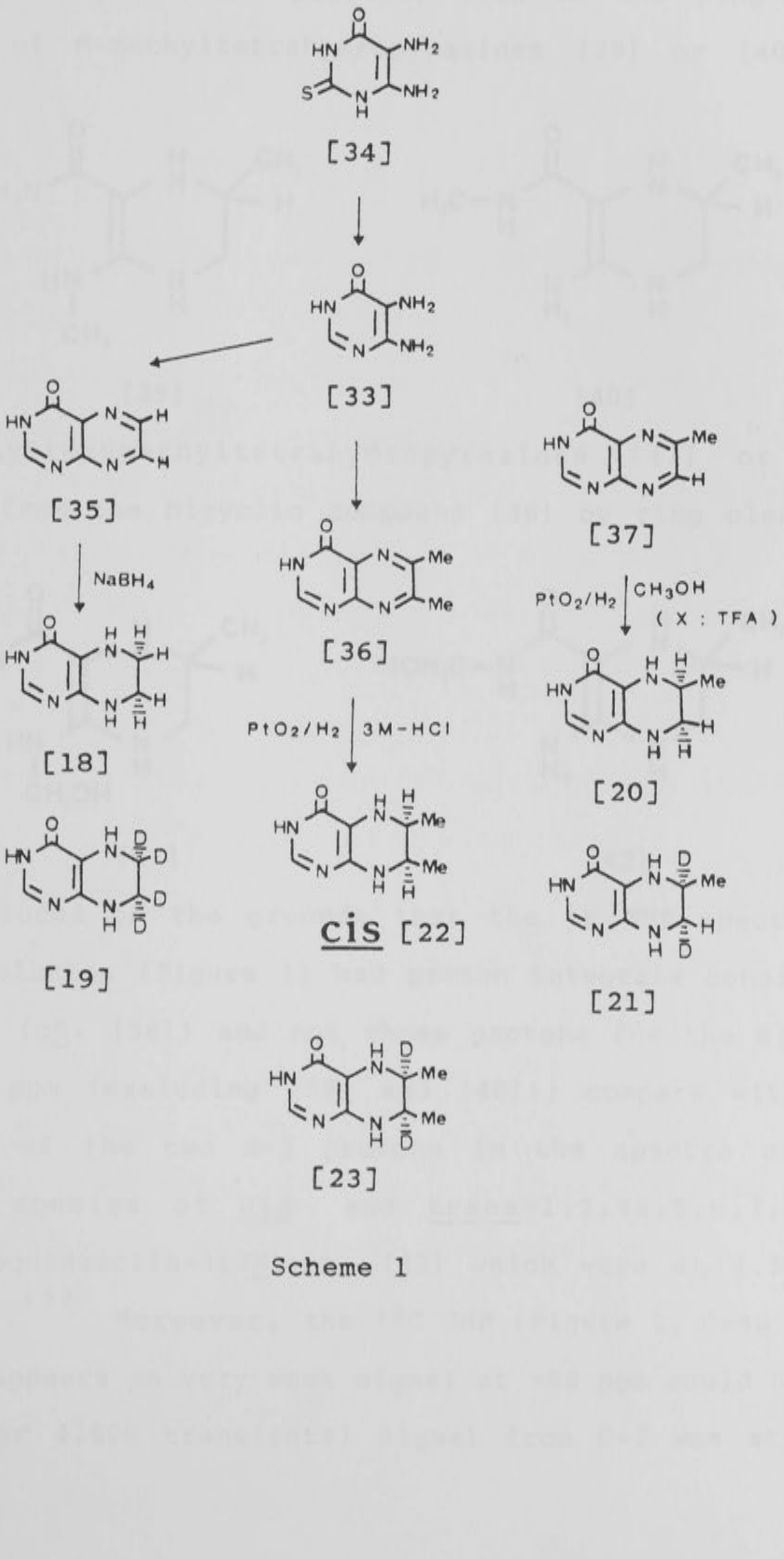
Pteridin-4(3H)-one [35] which was prepared by following the method of Albert et al.[127] could not be

reduced catalytically with pre-reduced platinum oxide in trifluoroacetic acid, or with Raney nickel in ethanol: This was found not to be due to poisoning of the catalyst by traces of sulphur from the intermediates because the reductions were unsuccessful with rigorously purified pteridinone, and when large excesses of catalyst were used. No satisfactory explanation for this failure could be presented other than severe poisoning of the catalysts by traces of reduced intermediates. Reduction to the tetrahydro derivative with sodium borohydride was, however, successful (cf. reference 128). Similarly [6,6,7,7-D_4]-5,6,7,8-tetradeuteriopteridin-4(3H)-one [19] was prepared by using [1,2-D_2]-glyoxal bis-(sodium bisulphite) to synthesize 6,7-dideuteriopteridin-4(3H)-one which was reduced with sodium borodeuteride in D_2O.

6-Methylpteridin-4(3H)-one [37],[129] on the other hand, gave the reduced derivatives [20] and [21] on hydrogenation with platinum oxide in methanol and by reduction with sodium borodeuteride in D_2O respectively. In the former case, however, when the reduction was carried out in trifluoroacetic acid and stopped after two equivalents of hydrogen were absorbed, a mixture of tetra-, [20], and hexa-, [38], hydropteridinone was produced.

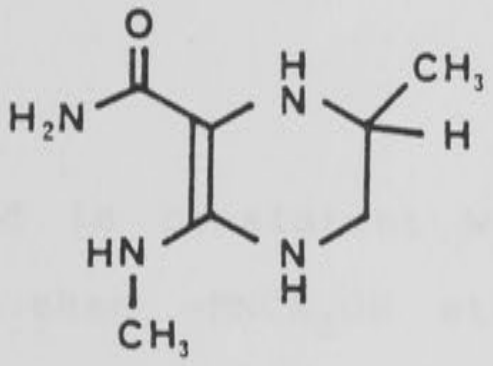


[38]

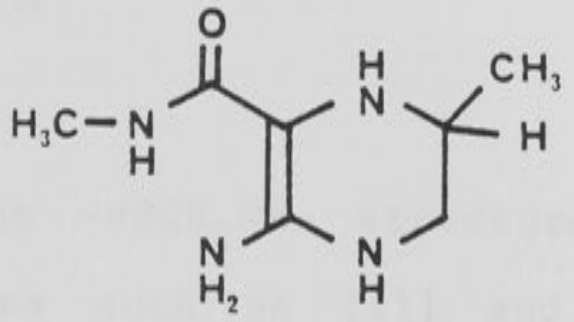

[34]
[33]
[35]
[37]
NaBH4
[36]
PtO2/H2
CH3OH
(X : TFA)
[18]
PtO2/H2
3M-HCl
[20]
[19]
cis [22]
[21]
[23]

Scheme 1

The pure hexahydro derivative [38] was obtained by allowing reduction to proceed to completion. Alternative structures for the fully reduced product, such as the ring-opened cations of N-methyltetrahydropyrazines [39] or [40], or

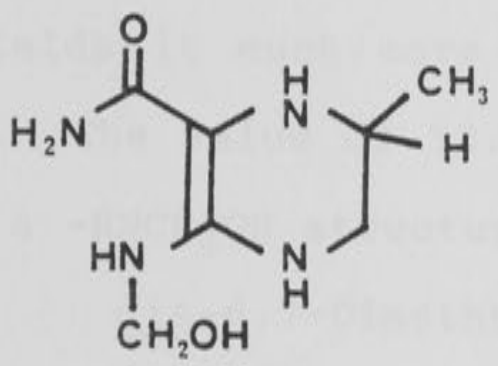


[39]

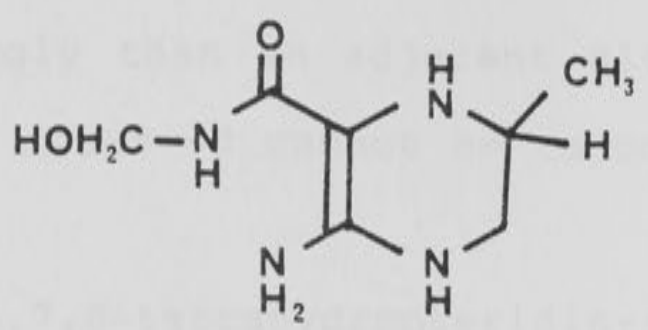


[40]

the N-hydroxymethyltetrahydropyrazines [41] or [42] derived from the bicyclic compound [38] by ring cleavage,

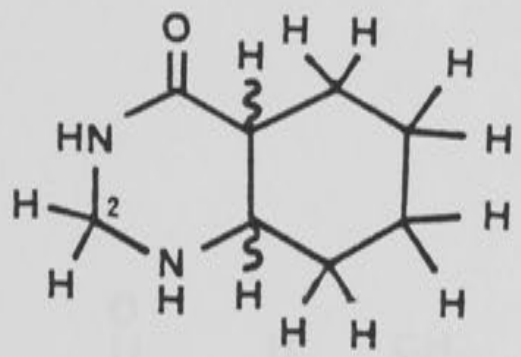


[41]



[42]

were excluded on the grounds that the ^{1}H NMR spectra in acidic solution (Figure 1) had proton integrals consistent with two (cf. [38]) and not three protons for the singlet at 4.99 ppm (excluding [39] and [40]); compare with the singlets of the two H-2 protons in the spectra of the neutral species of cis- and trans-1,2,4a,5,6,7,8,8a-octahydroquinazolin-4(3H)-one [43] which were at 4.30 and 4.33 ppm.[130] Moreover, the ^{13}C NMR (Figure 2, C-4a which usually appears as very weak signal at ~98 ppm could not be seen after 4,600 transients) signal from C-2 was at 52.1



[43]

ppm and is consistent with the $-HNCH_2NH-$ structure [38] rather than $-HNCH_2OH$ structures such as [41] and [42]. This deduction was made from the known ^{13}C chemical shifts of 6-aminohexan-1-ol where the signals from C-1 and C-6 were at 62.9 and 43.2 ppm respectively.[131] The model shows that the oxygen atom adjacent to a methylene carbon atom deshields it much more strongly than an adjacent nitrogen atom. The value of 52.1 ppm observed cannot be reconciled with a $-HNCH_2OH$ structure.

cis-6,7-Dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [22] was prepared as the hydrochloride salt by catalytic hydrogenation of the respective pteridinone in acidic medium and allowed to proceed to completion. The cis stereochemistry at C-6 and C-7 was deduced from the small H-6, H-7 coupling constants (cf. reference 132) in the ^1H NMR spectra (Figure 3). 6,7-Dimethyl-[6,7-D_2]-5,6,7,8-tetrahydropteridin-4(3H)-one [23] hydrochloride was similarly prepared by catalytic hydrogenation with D_2 gas.

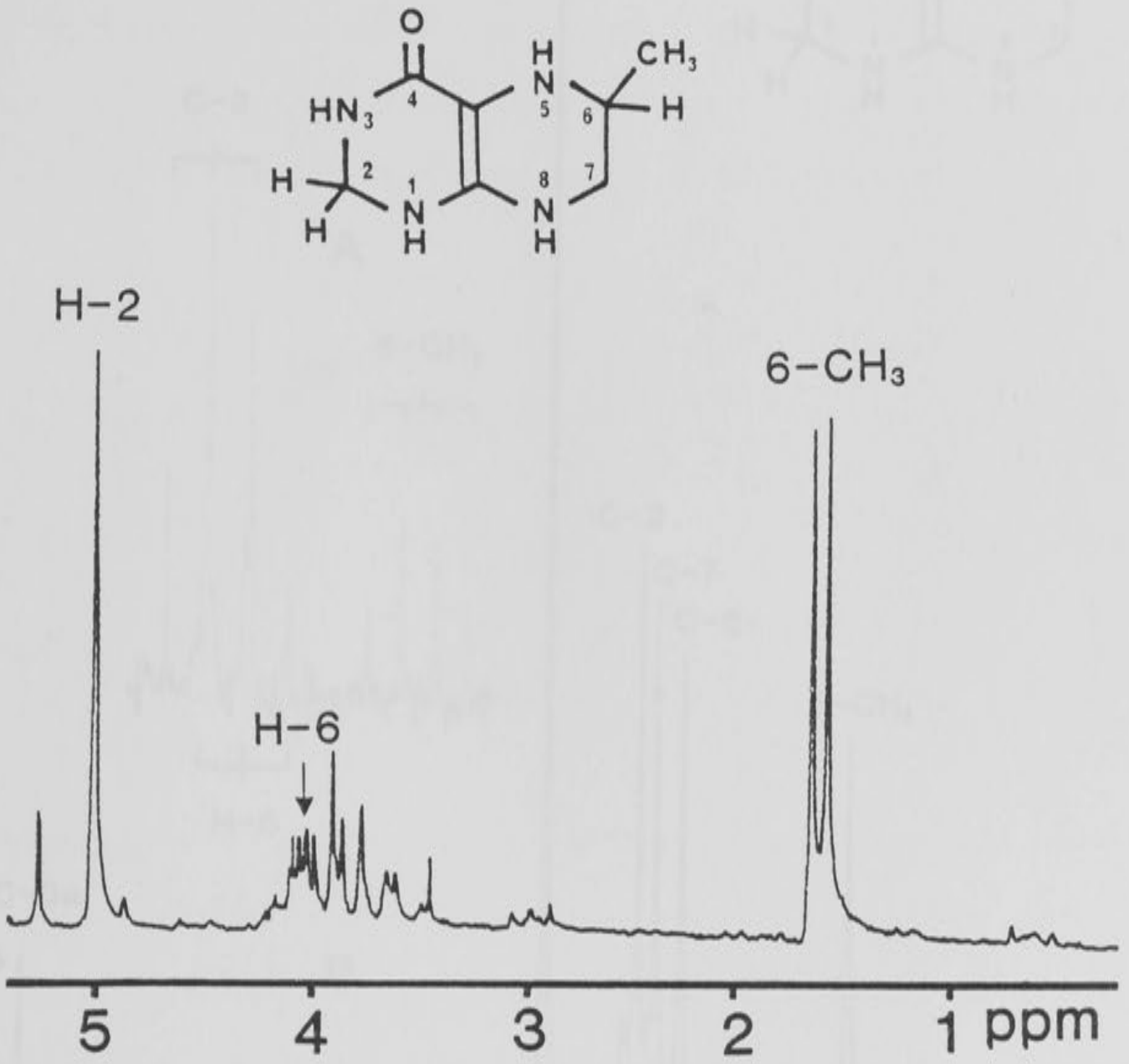


Figure 1. ^{1}H NMR spectrum of 6-methyl-2,3,5,6,7,8-hexahydropteridin-4(3H)-one [38] in 1 M DCl at 90 MHz.

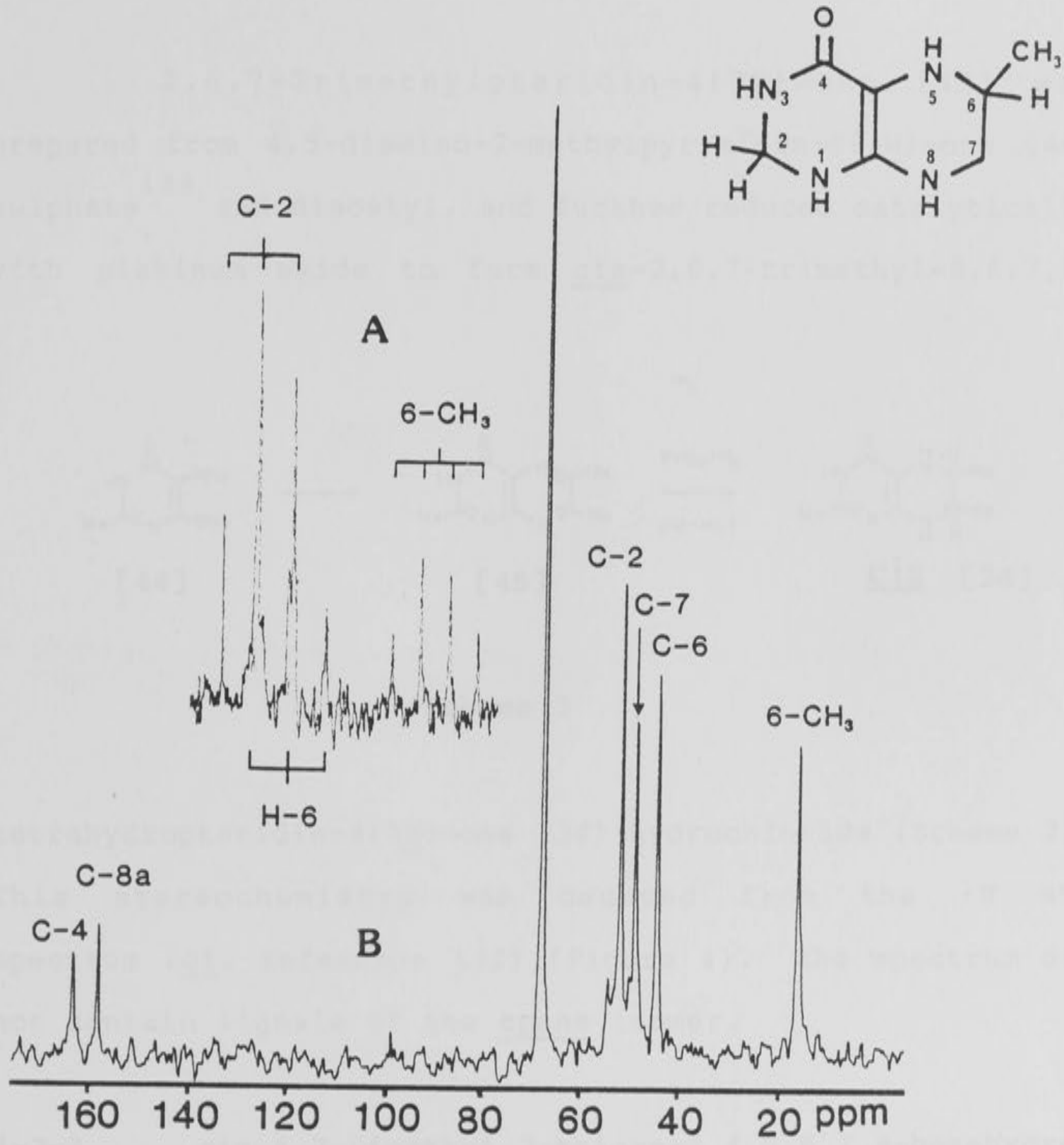
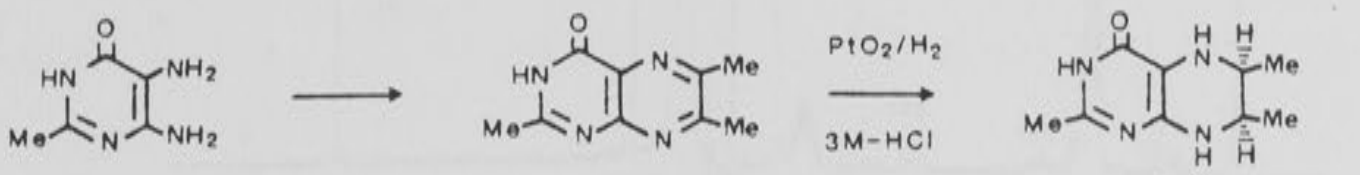


Figure 2. ^{13}C NMR spectrum of 6-methyl-2,3,5,6,7,8-hexahydropteridin-4(3H)-one [38] [A proton coupled (8,700 transients); B proton decoupled (4,600 transients)] in D_2O at 90 MHz.

3-2-2 cis-2,6,7-Trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [24]

2,6,7-Trimethylpteridin-4(3H)-one [45] was prepared from 4,5-diamino-2-methylpyrimidin-6(1H)-one [44] sulphate[133] and diacetyl, and further reduced catalytically with platinum oxide to form cis-2,6,7-trimethyl-5,6,7,8-



[44] [45] **cis** [24]

Scheme 2

tetrahydropteridin-4(3H)-one [24] hydrochloride (Scheme 2). This stereochemistry was deduced from the ^{1}H NMR spectrum (cf. reference 132) (Figure 4). The spectrum did not contain signals of the trans isomer.

3-2-3 cis-6,7-Dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one [27]

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one [46][134,135] (which was prepared by the condensation of 4,5-diamino-2-thioxopyrimidin-6(1H)-one and diacetyl), was reduced to the cis-hexahydro derivative [27] (see ^{1}H NMR spectrum, Figure 5) with excess sodium borohydride (Scheme

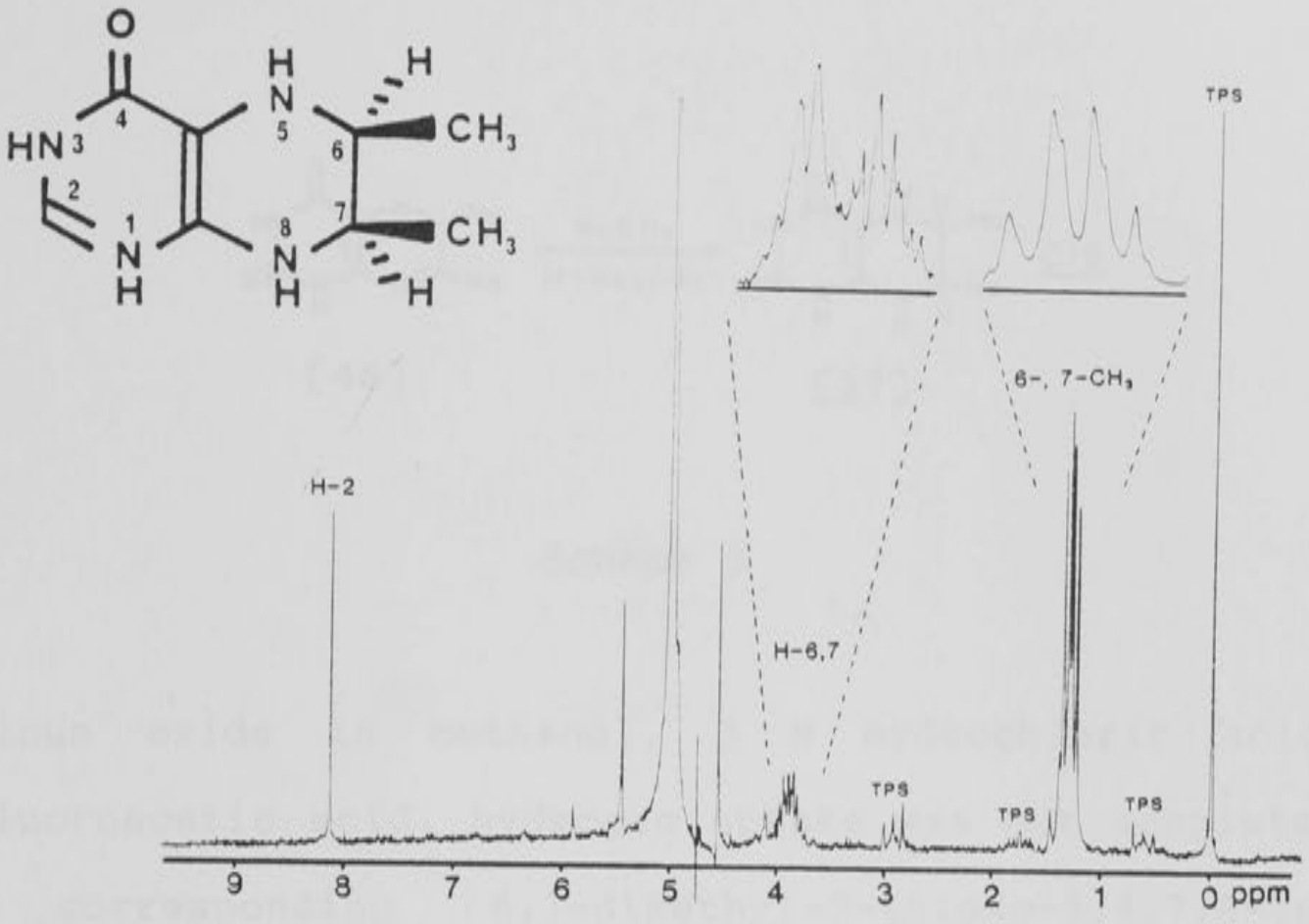


Figure 3. ^{1}H NMR spectrum of cis-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [22] in 0.5 M DCl at 90 MHz.

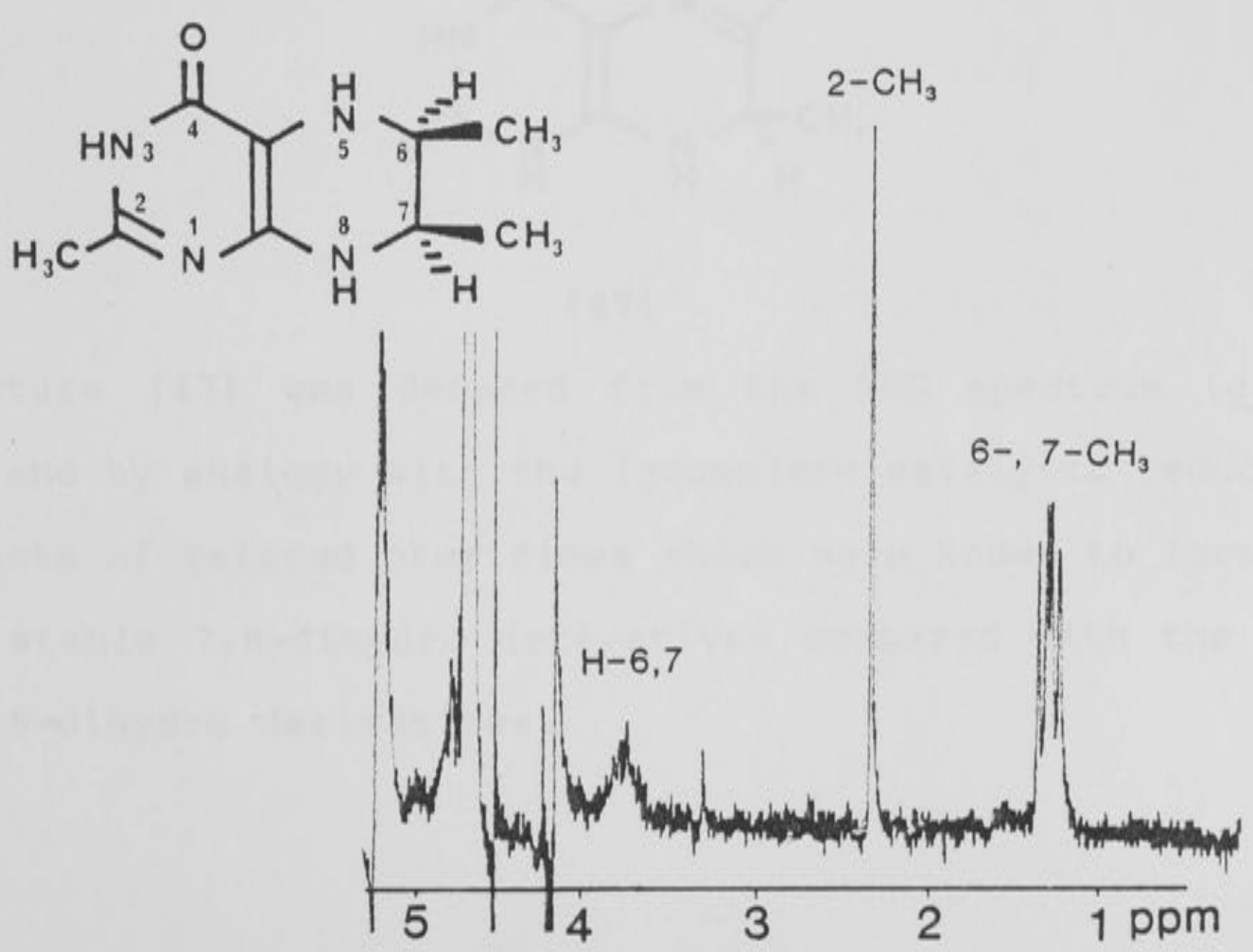
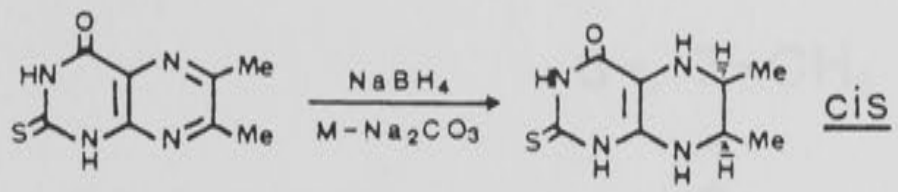


Figure 4. ^{1}H NMR spectrum of cis-2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [24] in D_2O at 90 MHz.

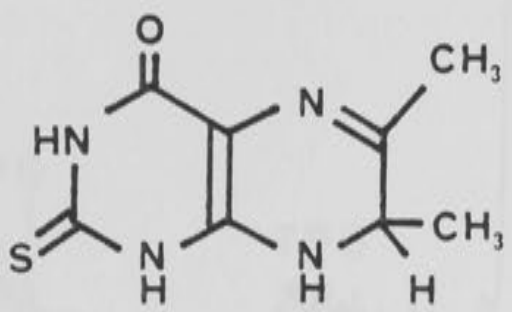
3). However, when it was reduced catalytically with



[46] [27]

Scheme 3

platinum oxide in methanol, 3 M hydrochloric acid or trifluoroacetic acid, hydrogen uptake was not complete and the corresponding 6,7-dimethyl-2-thioxo-3,4,7,8-tetrahydropteridin-4(1H)-one [47] was formed. This 7,8-dihydro



[47]

structure [47] was deduced from the NMR spectrum (cf. p 168) and by analogy with the incomplete catalytic reduction products of related pteridines which were known to form the more stable 7,8-dihydro derivatives compared with the 5,6- or 5,8-dihydro derivatives.

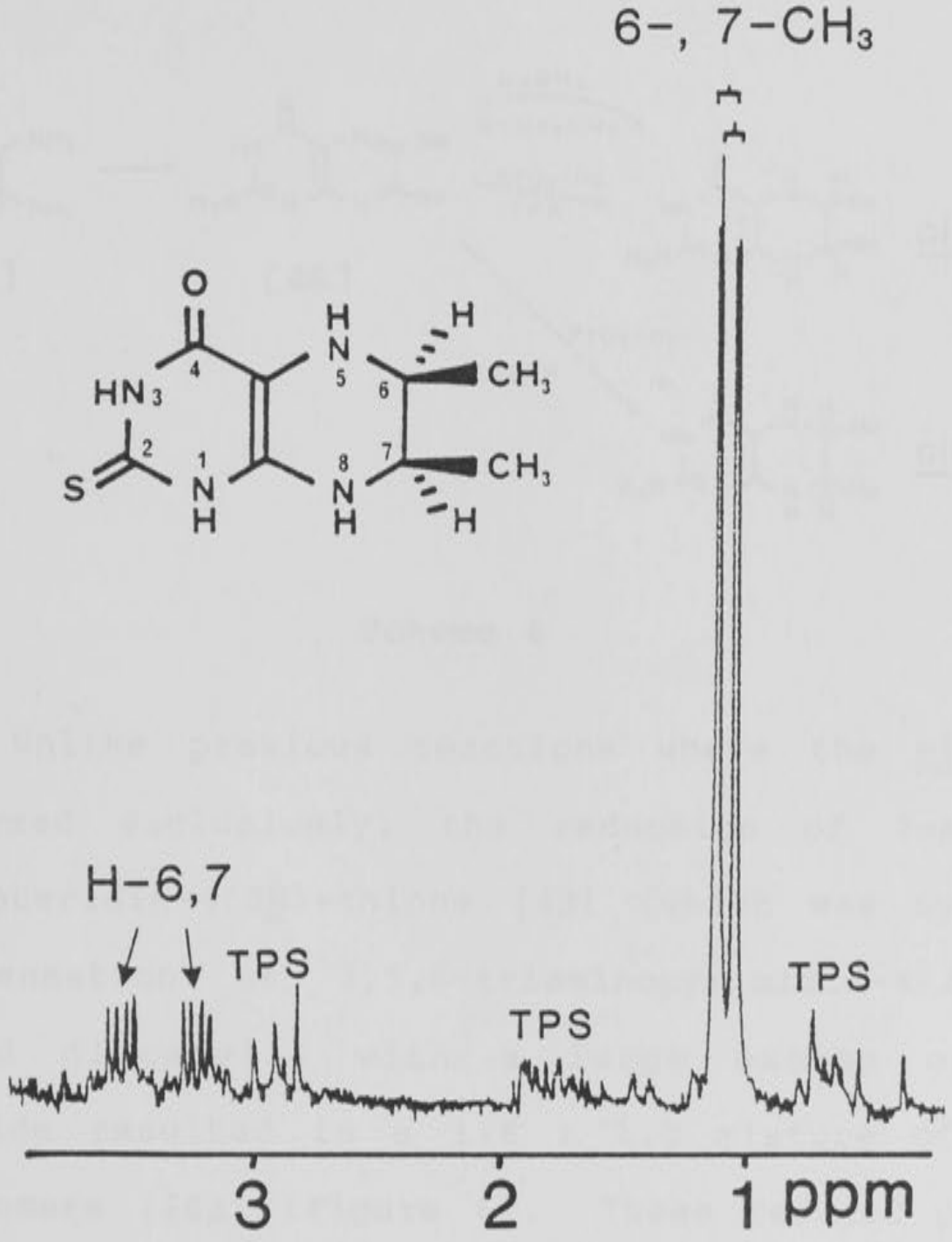
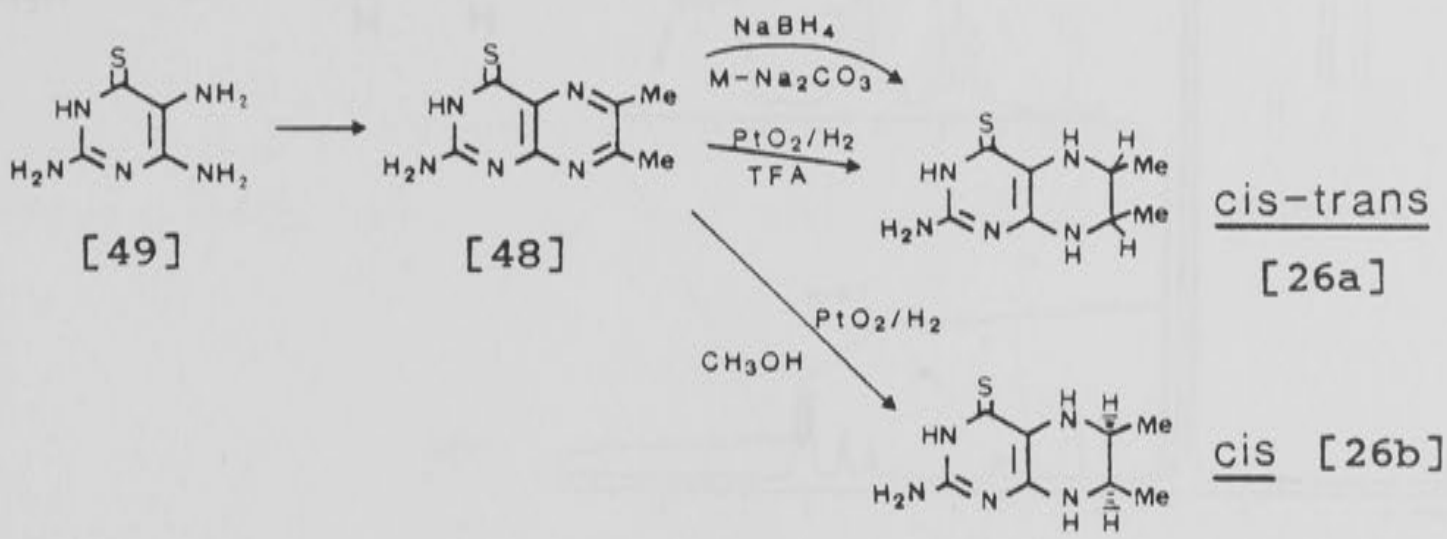


Figure 5. ^{1}H NMR spectrum of cis-6,7-dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one [27] in 0.01 M NaOD at 90 MHz.

3-2-4 2-Amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26] (Scheme 4)



Scheme 4

Unlike previous reactions where the cis-isomers were formed exclusively, the reduction of 2-amino-6,7-dimethylpteridin-4(3H)-thione [48] (which was synthesized by condensation of 2,5,6-triaminopyrimidin-4(3H)-thione [49] and diacetyl), with a large excess of sodium borohydride resulted in a 1.6 : 1.0 mixture of cis and trans isomers [26a] (Figure 6). These reduced pteridines could not be obtained completely free from borate and the stereo isomers were not separated. This mixture of cis and trans isomers was used for the enzymic assays. In an attempt to obtain preparations free from borate the 2-amino-6,7-dimethylpteridin-4(3H)-thione [48] was reduced catalytically with platinum oxide in methanol. This gave only the cis-5,6,7,8-tetrahydro derivative [26b] (by ^{1}H NMR) (Figure 7). Similar reduction in trifluoroacetic acid

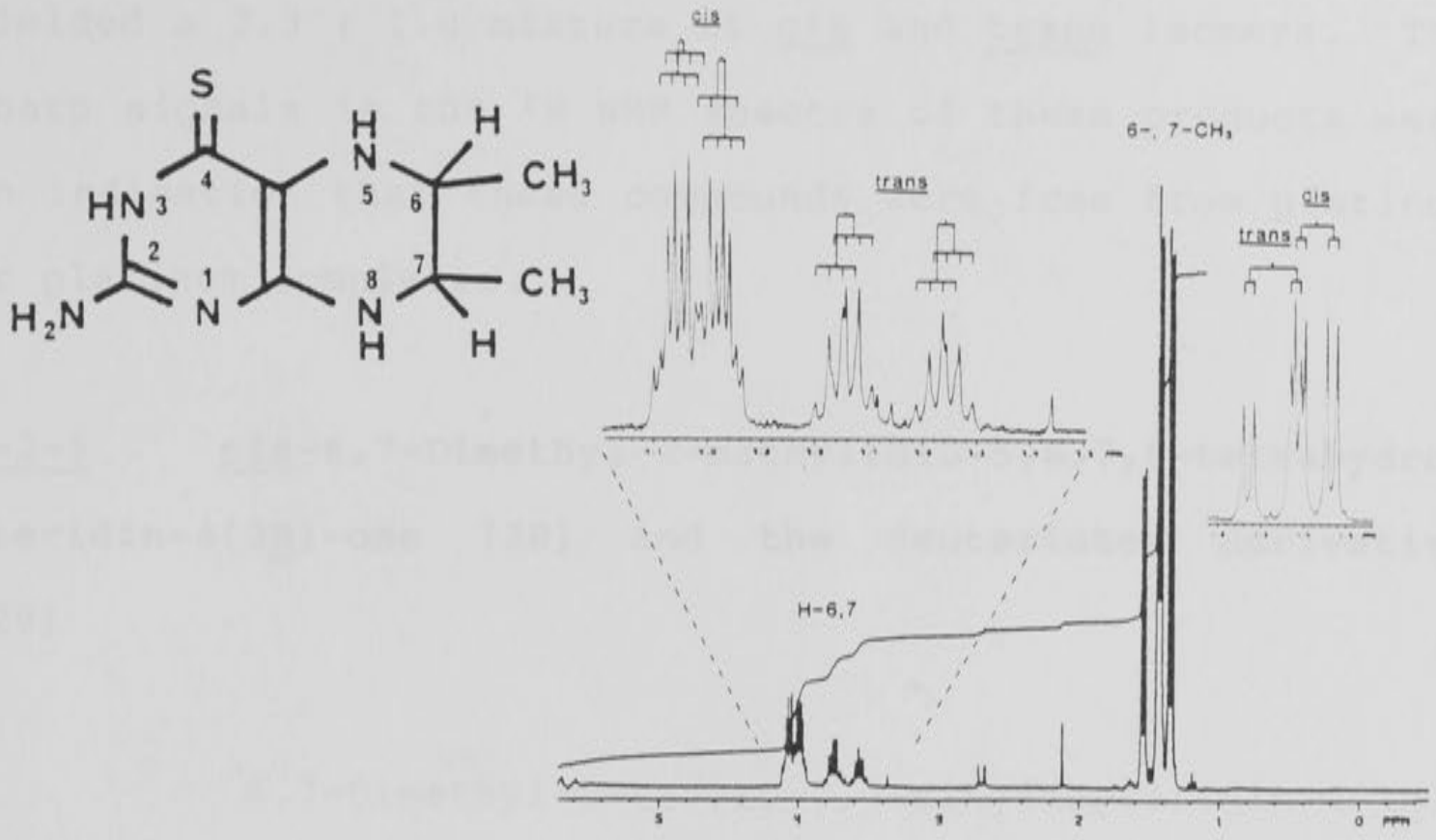


Figure 6. ^{1}H NMR spectrum of the mixture of cis- and trans-2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26a] in 2 M DCl at 270 MHz.

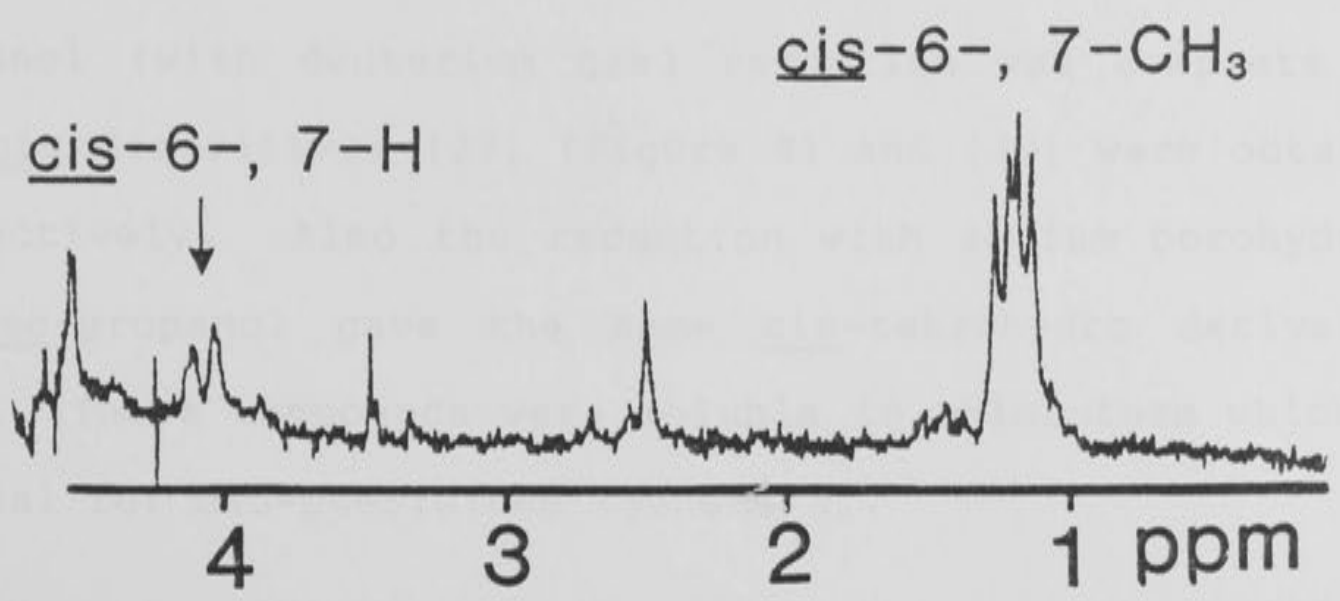


Figure 7. ^{1}H NMR spectrum of cis-2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26b] hydrochloride in D_2O at 90 MHz.

yielded a 2.3 : 1.0 mixture of cis and trans isomers. The sharp signals in the ^{1}H NMR spectra of these products were an indication that these compounds were free from platinum or platinum complexes.

3-2-5 cis-6,7-Dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one [28] and the deuteriated derivative [29]

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one [46] (see Section 3-2-3, p 104) reacted with methyl iodide and formed 6,7-dimethyl-2-methylthiopteridin-4(3H)-one [50]. This preparation of the 2-methylthio derivative [50] was different from before.[134,136] It had been previously prepared by the condensation of 2-methylthiopyrimidin-4(3H)-one and diacetyl. Catalytic hydrogenation of this 6,7-dimethyl-2-methylthiopteridin-4(3H)-one [50] with platinum oxide in trifluoroacetic acid gave 7,8-dihydro derivative. When the solvent was methanol or (D_4)-methanol (with deuterium gas) reduction was complete and the cis derivatives [28] (Figure 8) and [29] were obtained respectively. Also the reduction with sodium borohydride in iso-propanol gave the same cis-tetrahydro derivative [28]. These compounds were soluble in chloroform which is unusual for oxo-pteridines (Scheme 5).

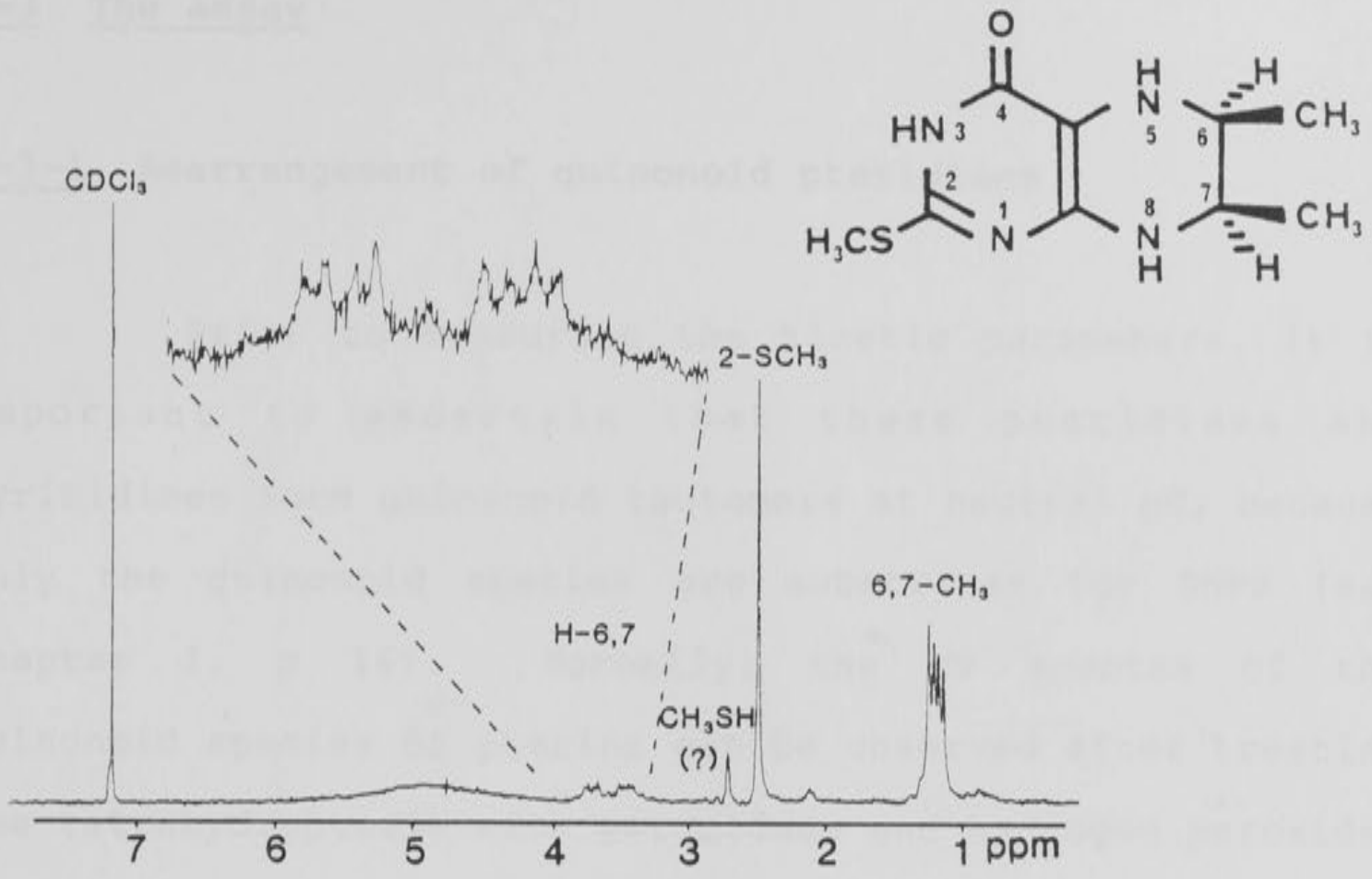
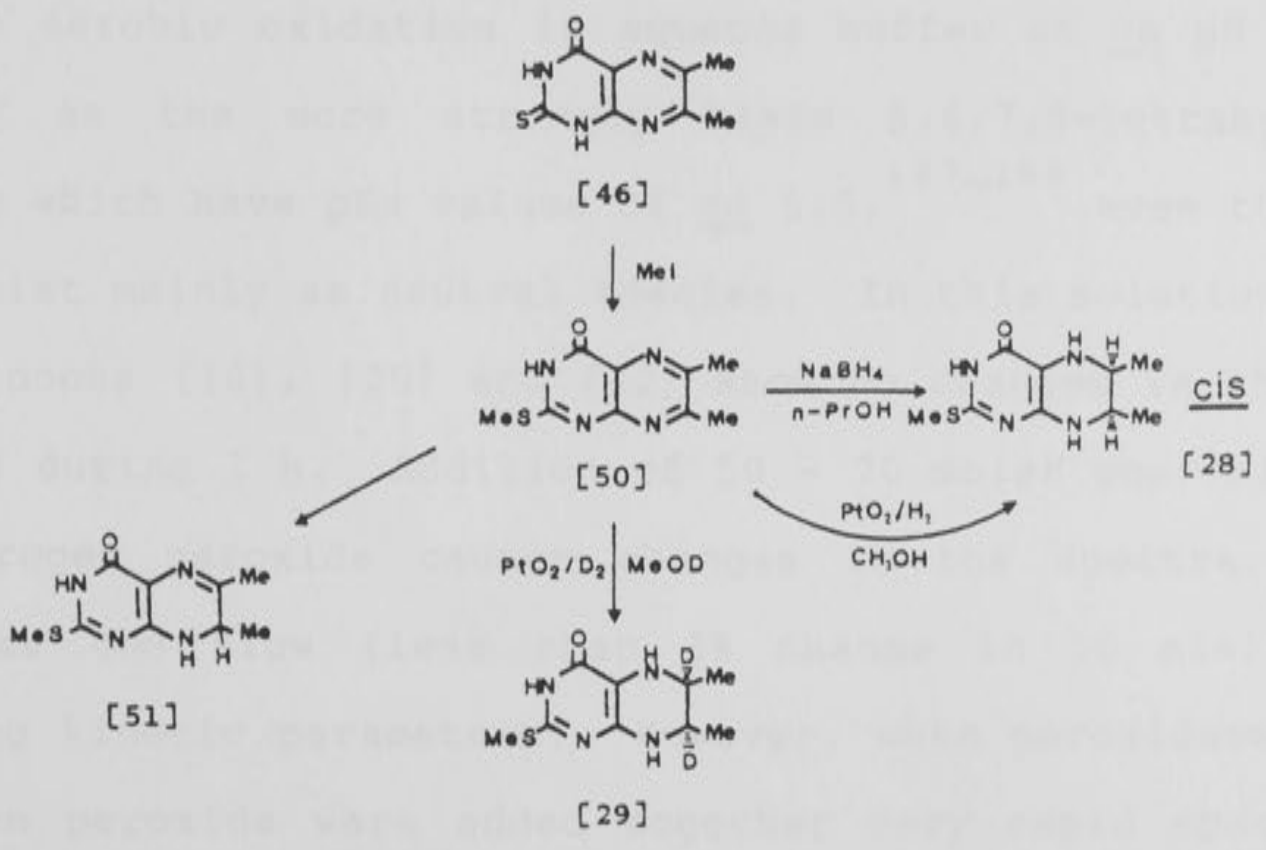


Figure 8. ^{1}H NMR spectrum of cis-6,7-dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one [28] in $CDCl_3$ at 90 MHz.



Scheme 5

3-3 The assay

3-3-1 Rearrangement of quinonoid pteridines

Prior to measuring the kinetic parameters, it is important to ascertain that these pteridines and pyrimidines form quinonoid tautomers at neutral pH, because only the quinonoid species are substrates for DHPR (see Chapter 1, p 16). Normally, the UV spectra of the quinonoid species of pterins can be observed after treating the tetrahydropterin with peroxidase and hydrogen peroxide. These quinonoid species are not stable and rearrange to the respective 7,8-dihydro(3H) compounds (tautomers) which are not substrates any more for DHPR (Figure 9).[22]

The 5,6,7,8-tetrahydropteridin-4(3H)-ones [18], [20] and [22] which have pKa values of ca 3.8[128] do not undergo aerobic oxidation in aqueous buffer at ca pH 7 as readily as the more strongly basic 5,6,7,8-tetrahydropterins which have pKa values of ca 5.5,[137~140] even though they exist mainly as neutral species. In this solution the pteridinones [18], [20] and [22] show no changes in the UV spectra during 1 h. Addition of 50 - 70 molar equivalents of hydrogen peroxide causes changes in the spectra, but they are too slow (less than 5% change in 10 min) for deriving kinetic parameters. However, when peroxidase and hydrogen peroxide were added together very rapid spectral changes occurred (Figure 10). The spectral changes for each compound were slightly different, but all

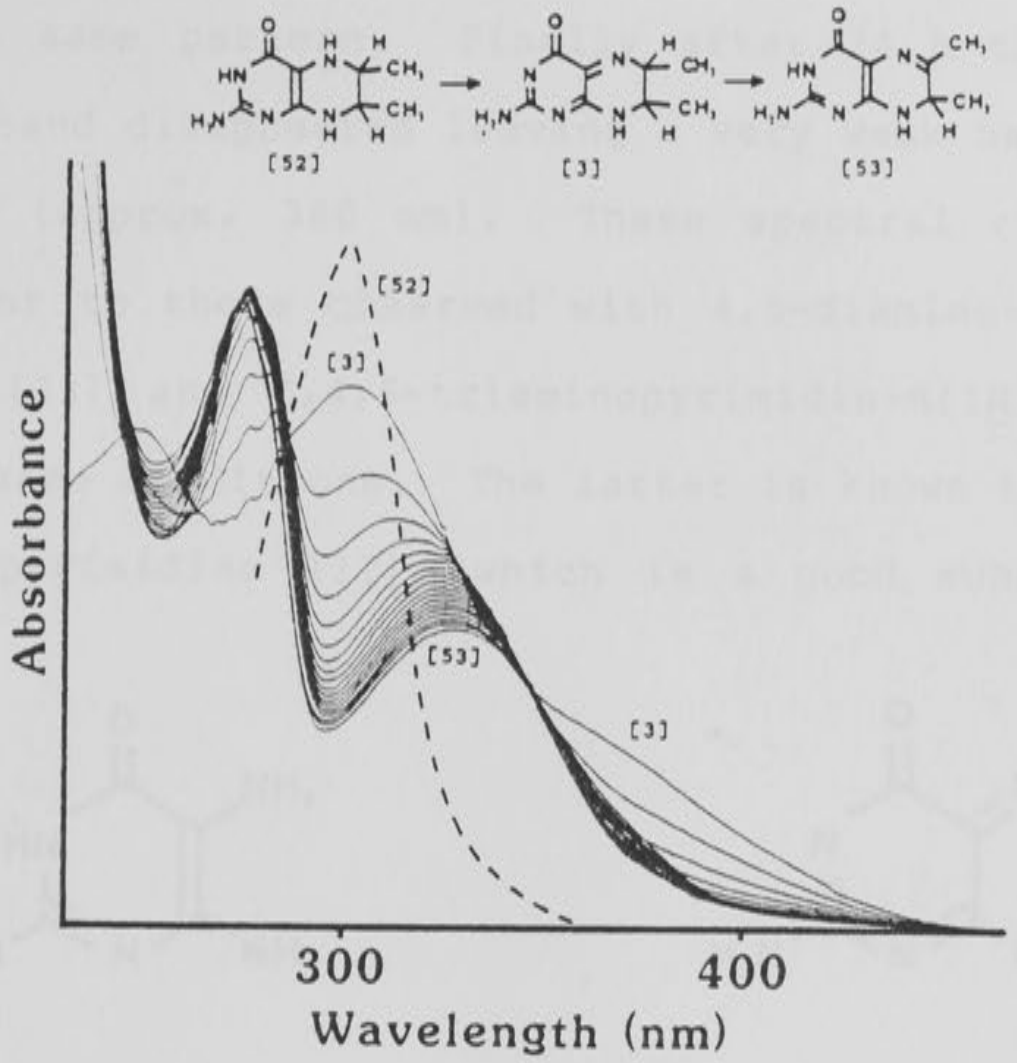


Figure 9. UV spectra changes of 6,7-dimethyl-5,6,7,8-tetrahydropterin [52].

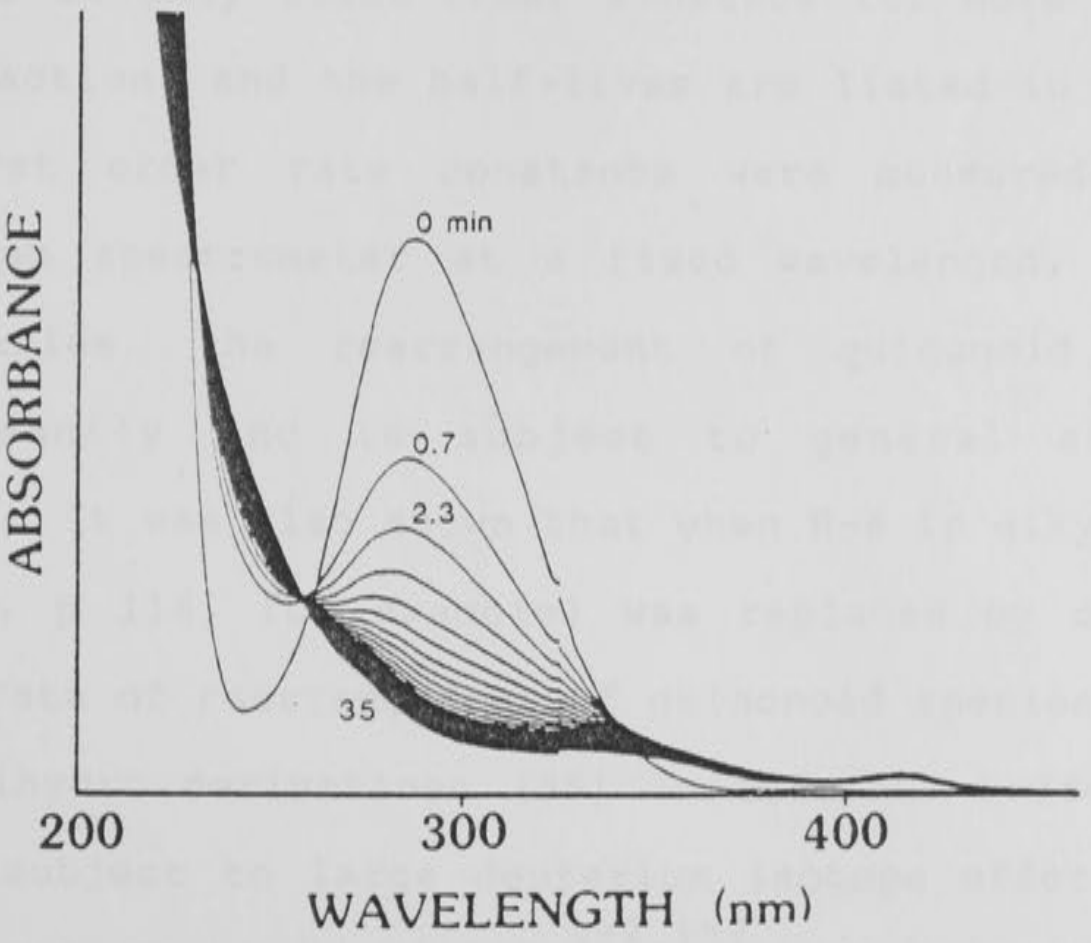
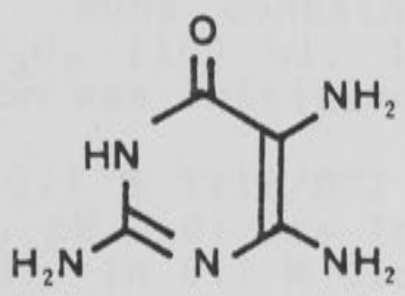
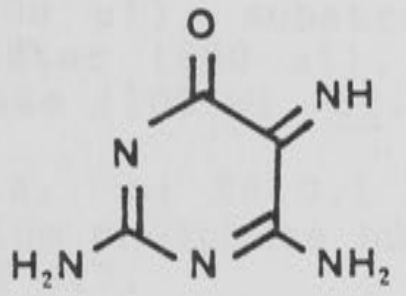


Figure 10. UV spectra of 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one [20] after treatment with peroxidase and hydrogen peroxide in 0.1 M Tris/HCl buffer, pH 7.4 at 25^{0}C.

showed the same pattern. Finally after 24 h the main UV absorbing band disappeared leaving a very weak band at long wavelength (approx. 380 nm). These spectral changes are very similar to those observed with 4,5-diamino-pyrimidin-6(1H)-one [33] and 2,4,5-triaminopyrimidin-6(1H)-one [54] under the same conditions. The latter is known to give the quinonoid pyrimidine [12], which is a good substrate for



[54]



[12]

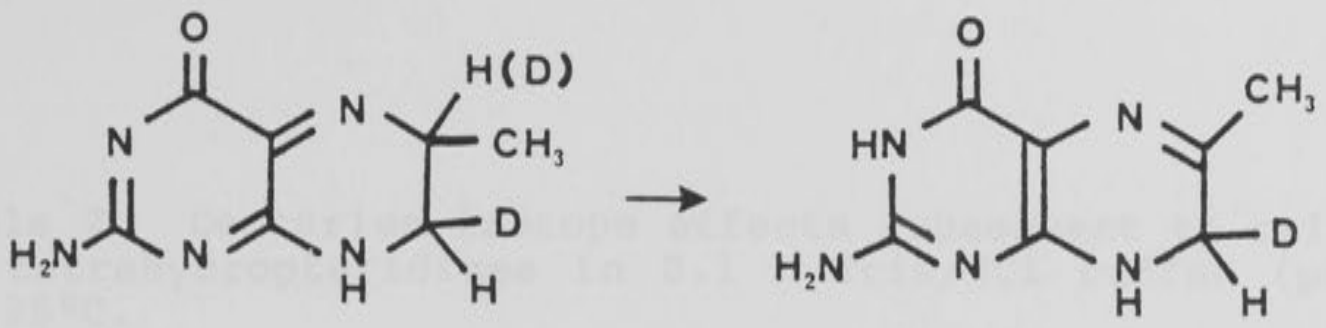
DHPR.[141] The spectral changes of the quinonoid pteridin-4(6H)-ones (rearrangement and decomposition, cf. Figure 10) were found to obey first-order kinetics for more than 85% of the reaction, and the half-lives are listed in Table 1. These first order rate constants were measured with a single beam spectrometer at a fixed wavelength. In the pterin series, the rearrangement of quinonoid species occurs readily and is subject to general acid-base catalysis. It was also shown that when H-6 in alkylpterins (Scheme 6, p 116, for example) was replaced by deuterium then the rate of rearrangement of quinonoid species [55] to the 7,8-dihydro derivatives [56] in Tris/HCl buffer at ca pH 7 was subject to large deuterium isotope effects (i.e. k_H/k_D values as large as 10).[142,143] The respective 6,7-dideuteriated pteridinone derivatives of [18], [20] and

Table 1. First order rate constants for the changes subsequent to oxidation of compounds, [18], [20], [22] and [33], at 25°C.

Runs contained: buffer (100 µl), substrate (100 µl), H_2O_2 (100 µl, 11 µmol) and water (600 µl), and the reaction was initiated with peroxidase (100 µl, cf. p 174).

a: In 0.1 M Tris/HCl buffer, pH 7.4; b: In 0.1 M Bicine buffer, pH 7.0; c: In 0.3 M potassium phosphate buffer, pH 7.1; d: In 0.1 M Bicine buffer, pH 6.7.

5,6,7,8-tetra-hydropteridin-4(3H)-one	buffer	$k_{obs} \times 10^3$ (sec^{-1})	$t_{1/2}$ (min)	conc. (µM)	$\lambda_{analyt.}$ (nm)
Unsubstituted [18]	a	5.0(±0.08)	2.3	149	289
6-Methyl- [20]	a	2.1(±0.02)	5.6	143	290
	b	1.9(±0.02)	6.0	117	290
	c	0.8(±0.01)	13.9	117	290
6,7-Dimethyl- [22]	a	2.1(±0.04)	5.5	172	292
	b	1.2(±0.01)	9.6	166	292
	c	0.4(±0.01)	29.4	166	292
4,5-Diamino-pyrimidin-6(1H)-one [33]	a	6.1(±0.01)	1.9	282	190
	d	8.2(±0.01)	1.4	282	190
	c	7.1(±0.08)	1.6	282	190



[55] [56]

Scheme 6

[22], i.e. [19], [21] and [23] were synthesized and the UV spectral changes immediately after oxidation with peroxidase and hydrogen peroxide were examined. These were identical with those of the respective protio compounds (cf. Figure 10, p 113). Direct comparison revealed no deuterium isotope effects (Table 2), and the quinonoid dihydro(6H)pteridinones formed clearly did not rearrange smoothly to the corresponding 7,8-dihydro(3H) derivatives as occurred in the respective pterins. The absence of isotope effects from the measurements of compounds [18] to [23] indicates that after oxidation to the quinonoid species these pteridinones are undergoing decomposition more rapidly than the usual rearrangement. The observed spectral changes for the pyrimidinone [33] were faster in comparison (cf. Table 1). The spectral changes observed after oxidation of compounds [18], [20], [22] and [33] were faster in Tris/HCl buffer (pH 7.3) than in Bicine buffer (pH 7.0) or in phosphate buffer (pH 7.0) (Table 1), whereas in general pterin derivatives rearranged faster in phosphate buffer than in Tris buffer.[143,144] These

Table 2. Deuterium isotope effects subsequent to oxidation of tetrahydropteridines in 0.1 M Tris/HCl buffer (pH 7.4) at 25°C.

	10^3 k_H/min^{-1}	10^3 k_D/min^{-1}	k_H/k_D
5,6,7,8-tetra-[a] hydropteridin-4(3H)-ones			
Unsubstituted [18]	297	371	0.8
6-Methyl- [20]	124	126	1.0
6,7-Dimethyl- [22]	127	139	0.9
2-Methylthio-6,7-dimethyl- [28]	0.75	0.04	18.8
5,6,7,8-tetra-[b] hydropterins			
2-Amino-* 6-methyl-	21.3	2.18	9.8
6,7-dimethyl- [52]	58.7	6.30	9.3
6,7,7-trimethyl-	38.9	3.35	11.6

* These values are from reference 22

a Only traces of quinonoid 7,8-dihydropteridin-4(6H)-ones are formed in these cases.

b 7,8-Dihydro(6H)pterins are formed almost exclusively.

differences could be expected because in the case of compounds [18], [20] and [22] the decompositions (in which cleavage of the C-H(D) bond at C-6 is not rate limiting) were the major contributors to the observed rates and not the rearrangements.

When the experiment was extended to a variety of substituted pteridines, [24] to [32], the respective quinonoid species were formed by oxidation with peroxidase and hydrogen peroxide or potassium ferricyanide (3 ~ 10 equivalents). Potassium ferricyanide was used as oxidant for DHPR assays in these cases, because the oxidation of 2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [24] with peroxidase and hydrogen peroxide was relatively slow, whereas the oxidation with three molar, or more, equivalents of potassium ferricyanide was complete within five seconds. All of these compounds [24] to [32] behaved more or less like the pterins on oxidation. They rearranged to the 7,8-dihydro(3H) tautomers (as observed by UV spectroscopy, cf. Figure 9, p 113) which were then further oxidized to pterins. However, in some of them, e.g. compounds [24], [26], [27], [28] and [29] the rearrangements of the quinonoid species were accompanied by some slight decomposition (e.g. Figure 11 compare with Figure 9 and 10). The decompositions were not very serious for the enzyme assay in these examples, because they occurred after the formation of quinonoid species and were slower than in the above pteridin-4-ones. The quinonoid species from compounds [25], [28] and [31] had typical UV

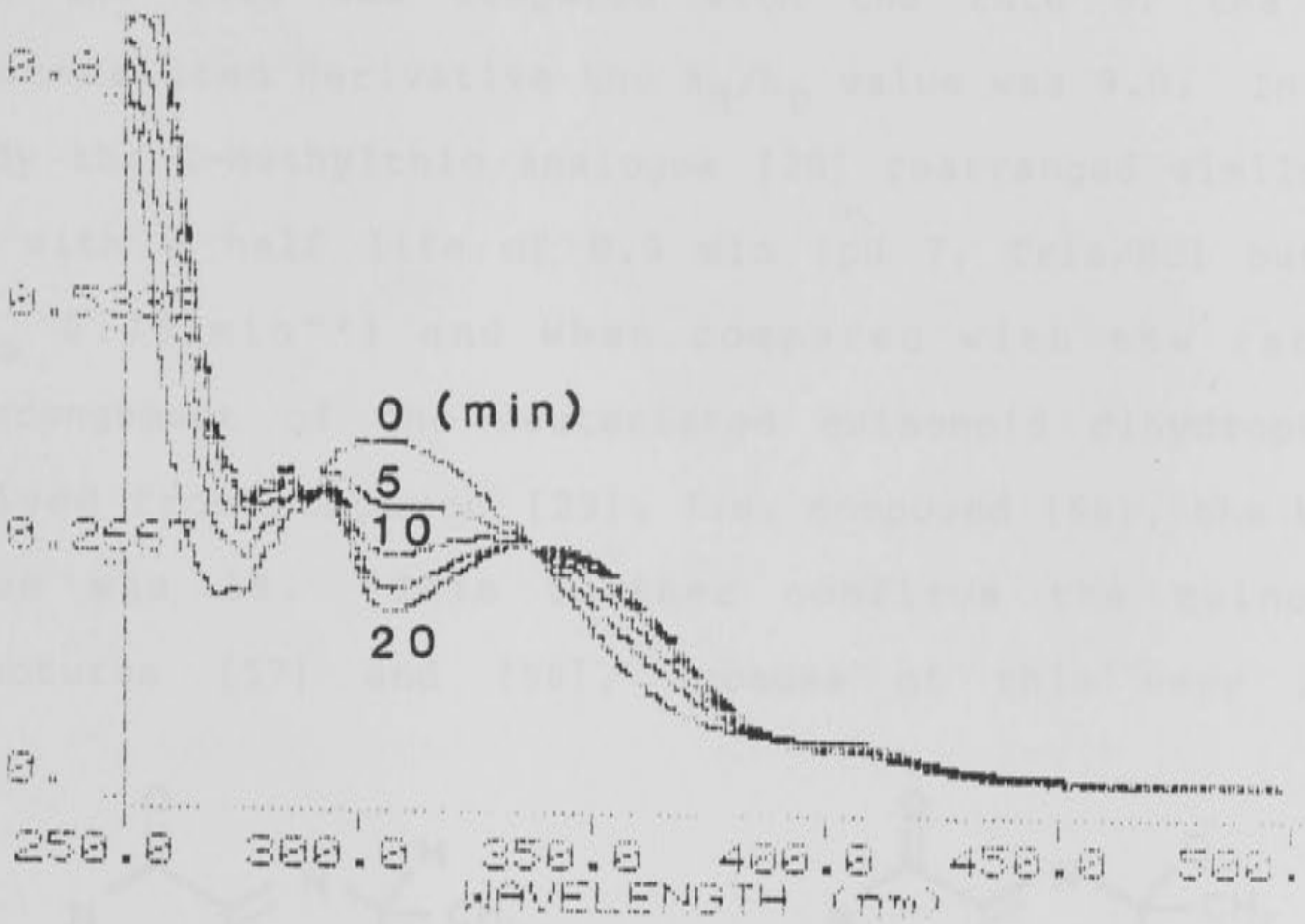
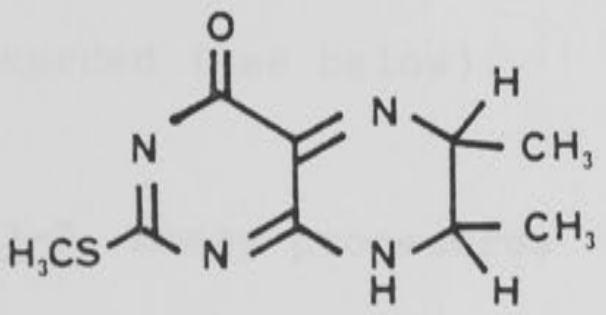
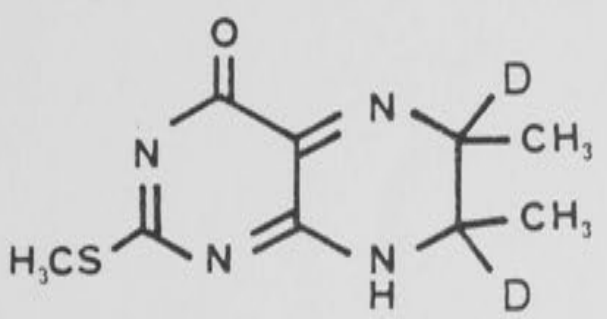


Figure 11. UV spectral changes of cis-6,7-dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one [28] with peroxidase and hydrogen peroxide (11.2 μM) at pH 7.3, 25°C.

spectra of quinonoid dihydropterins (as in Figure 9). Armarego and Waring[72] showed previously that the quinonoid species from the 2-methylamino derivative [25] rearranged to the 7,8-dihydro(3H) tautomer with a half life of 34.6 min (pH 7.5, Tris/HCl buffer, k_{obs} 2.0 x 10^{-2} min^{-1}), and when the rate was compared with the rate of the 6,7-dideuteriated derivative the k_H/k_D value was 9.0. In this study the 2-methylthio analogue [28] rearranged similarly, but with a half life of 0.9 min (pH 7, Tris/HCl buffer, k_{obs} 0.75 min^{-1}) and when compared with the rate of rearrangement of the deuteriated quinonoid dihydropterin derived from compound [29], i.e. compound [58], the k_H/k_D value was 18. This further confirms the quinonoid structures [57] and [58], because of this very large



[57]



[58]

deuterium isotope effect (cf. reference 22). The isotope effect, however, is much larger than the values usually observed with pterins, i.e. k_H/k_D ~10, suggesting that the protio compound was not undergoing exactly the same reactions at the same time as the deuterio compound.

3-3-2 Non-enzymic oxidation of NADH

NADH is oxidized non-enzymically by quinonoid dihydropterins. The rate of this reaction must be excluded from the enzymic rate. When the quinonoid species of compounds [18], [20], [22], and [24] to [32] were subjected to this study the rates of non-enzymic oxidation of NADH by these quinonoid species were only 1.4 ~ 6.8% of the enzymic rates (Table 3). It was shown that at the highest concentration of potassium ferricyanide the rate of oxidation of NADH by this iron complex was negligible (<0.05%) compared with the enzymic rate. The assay procedures, however, were such that the non-enzymic oxidation of NADH was compensated in the two cuvettes (in a double beam spectrometer), and only the enzymic rates were recorded (see below).

3-3-3 Assay procedures

In the first enzymic experiments the peroxidase and hydrogen peroxide oxidation products of compounds [18], [20], [22] and [33] were examined for inhibition of DHPR. It was found that no inhibition occurred at concentrations of 120 ~ 140 μM of the products when quinonoid 6-methyl-7,8-dihydro(6H)pterin [2] (p 92) (at 49 μM) and NADH (at 58 μM) were used. Preliminary studies showed that the oxidation products of each of the compounds [18], [20], [22] and [33] were substrates for DHPR.

Table 3. Non-enzymic oxidation of NADH at 25°C.

A: 0.1 M Tris/HCl buffer (pH 7.4), peroxidase, hydrogen peroxide (11 µmol); B: 0.1 M Tris/HCl buffer (pH 7.6), peroxidase; C: 0.1 M Tris/HCl buffer (pH 7.6), oxygen; D: 0.1 M Tris/HCl buffer (pH 7.4), potassium ferricyanide.

Compound	concn. (µM)	buffer	NADH (µM)	v_0 * 10^{-3} (u*/ml)
[18]#	149	A	77	>15.3
[20]#	143	A	77	> 7.7
[22]#	172	A	77	> 5.9
[33]#	190	A	77	>25.3
[24]	61	D	68	16.0
[25]∴	100	C	150	1.3
[26]∵	—	–	—	—
[27]	47	B	109	5.8
[28]	70	D	126	63.0
[29]	70	D	101	95.8
[32]	75	B	109	0.9
[52]	63	D	111	31.6

The rates were measured with a single beam spectrometer at 340 nm.

* µ moles NADH oxidized per min

\# Reaction was initiated after 1 min from the formation of quinonoid species.

∴ Values are from reference 72

∵ Values could not be measured because the spectral changes were not typical, i.e. the band at 340 nm increased with time.

The instability of the quinonoid species from compounds [18], [20], [22] and [33] made it difficult to use standard mixing procedures (see Chapter 1, p 20) because the decomposition of the initial quinonoid species formed can proceed to quite an extent in one cuvette before addition of an ingredient in the other cuvette. So the assay was performed by simultaneous addition of substrates and also simultaneous addition of NADH to both cuvettes in a double beam spectrophotometer. The order of addition of ingredients which gave best reproducibility of initial rate traces for a particular series of runs was selected (see Section 3-6-6, p 177). Because the initial products of peroxidase and hydrogen peroxide oxidation of the compounds [18], [20], [22] and [33] undergo decomposition readily, it was necessary to find out how the DHPR activity altered with time after allowing the decomposition to proceed before starting the assay for this enzyme. In this case the order of addition was Tris/HCl buffer, peroxidase, hydrogen peroxide, DHPR (to one cuvette), substrate (simultaneously to both cuvettes) and allowing a recorded time interval before initiating the reductase reaction by adding NADH (simultaneously to both cuvettes). A typical curve is shown in Figure 12. Maximum activity was observed at the shortest time. [In Figure 12, the concentration of hydrogen peroxide was very weak (11 μM) and any oxidation of DHPR by hydrogen peroxide should be minimal (see later, p 142).] This also suggests that the quinonoid species are formed rapidly and then

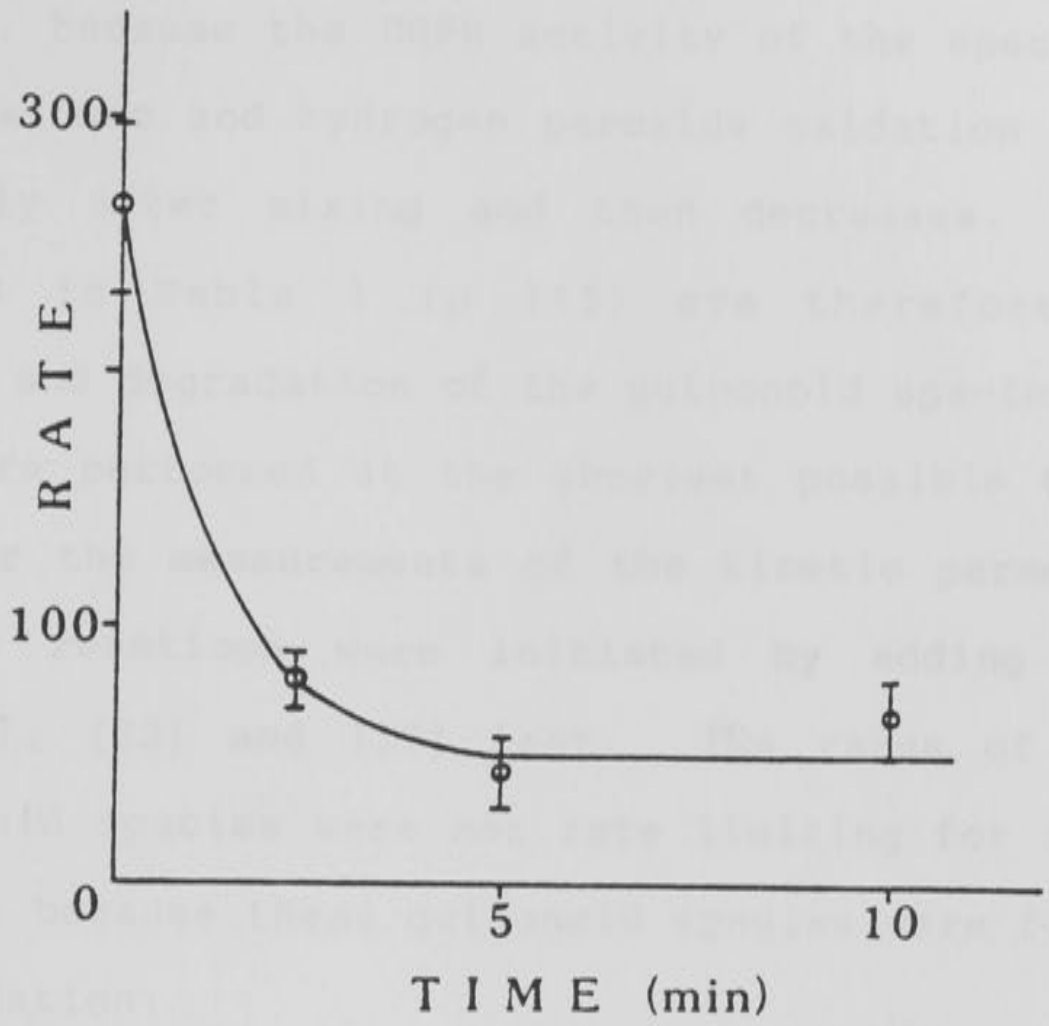


Figure 12. Dihydropteridine reductase activity at various time intervals after initiation of the peroxidase and hydrogen peroxide oxidation of 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one [20] and starting the reaction with NADH in 0.1 M Tris/HCl buffer (pH 7.4) at 25°C.

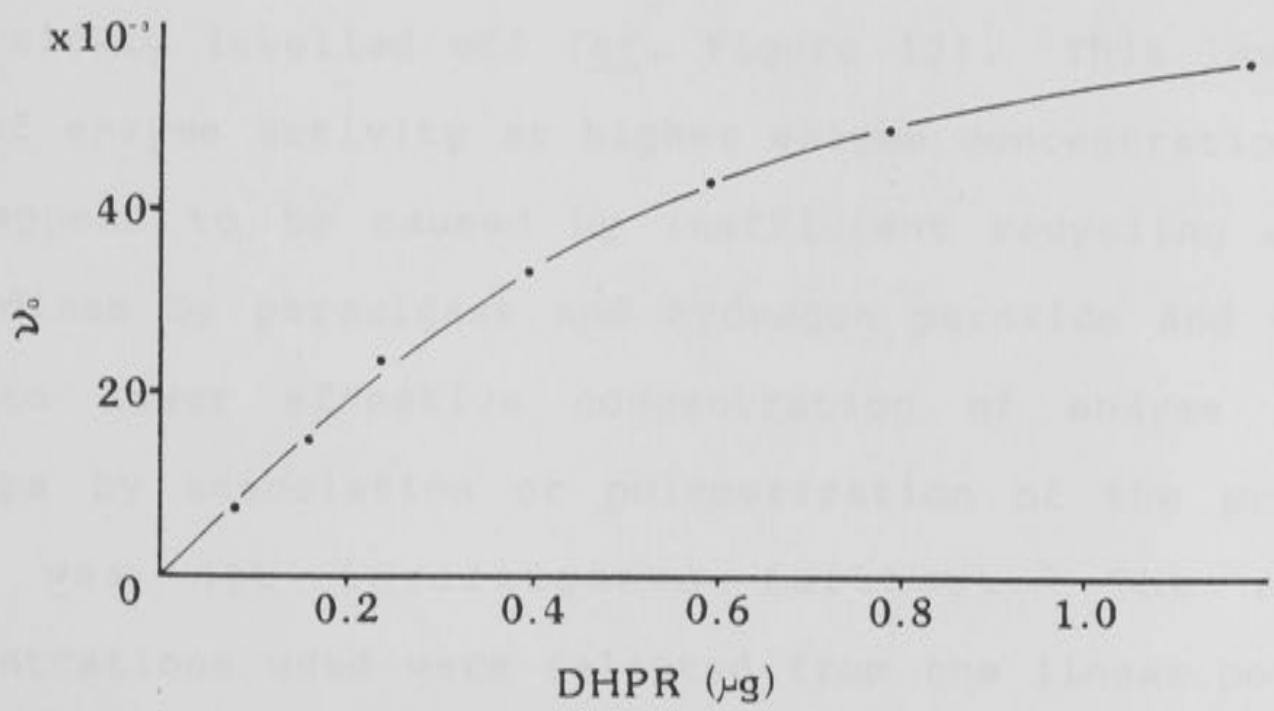


Figure 13. The effect of DHPR concentrations on the initial rates at pH 7.3, 25°C. 6-Methyl-5,6,7,8-tetrahydropteridin-4(3H)-one was used as a substrate precursor.

v_0 μmoles NADH oxidized / min per total protein.

decompose, because the DHPR activity of the species formed from peroxidase and hydrogen peroxide oxidation is highest immediately after mixing and then decreases. The rate constants in Table 1 (p 115) are therefore for the formation and degradation of the quinonoid species. So all assays were performed at the shortest possible time (ca 1 min). **For the measurements of the kinetic parameters for DHPR, the reactions were initiated by adding compounds [18], [20], [22] and [33] last.** The rates of formation of quinonoid species were not rate limiting for these four compounds, because these quinonoid species were formed soon after oxidation.

The concentrations of DHPR for the assays were selected from the plots of enzyme activity versus enzyme concentration. In the above cases the plots were linear up to enzyme concentrations of 0.4 ~ 0.6 µg/µl, i.e. the rate was doubled when the enzyme concentration was doubled, and then slowly levelled off (cf. Figure 13). This levelling off of enzyme activity at higher enzyme concentration does not appear to be caused by inefficient recycling of the pteridines by peroxidase and hydrogen peroxide and may be due to lower effective concentration of enzyme caused perhaps by association or polymerization of the protein. This was not investigated further. The enzyme concentrations used were selected from the linear portions of the plots (Table 4).

In the case of substituted pteridines, [24] to [32], the quinonoid substrates were generated by potassium

Table 4. DHPR assay conditions for each pteridine.

Pteridines	oxidants	enzyme [µl(µg)]	initiation by
[33]	A	1 (0.39)	pyrimidine
[18]	A	1 (0.39)	pteridine
[20]	A	7*(0.27)	pteridine
[22]	A	8*(0.31)	pteridine
[24]	B	8*(0.31)	NADH
[25]	B	4*(0.16)	NADH
[26a]	A	3 (1.17)	NADH
[27]	B	1 (0.39)	NADH
[28]	B	5*(0.19)	pteridine
[29]	B	5*(0.19)	pteridine
[30]	B	6*(0.24)	NADH
[31]	B	3*(0.12)	pteridine
[32]	B	3*(0.12)	pteridine
[52]	B	3*(0.12)	NADH

oxidants: A; peroxidase and hydrogen peroxide
B; potassium ferricyanide

* The diluted enzyme solution was used to inject larger aliquots (i.e. smaller error).

ferricyanide oxidation and the reaction was initiated as in Table 4. The type of oxidant did not affect the kinetic parameters as long as the formation of the quinonoid species was fast, i.e. not rate limiting.

3-3-4 Kinetic parameters with DHPR

Kinetic parameters for the pteridinones [15], [16] and [17].

The parameters for the pteridinones [15], [16] and [17] (p 95) are listed in Table 5. Although it was found that the quinonoid species of the 4,5-diaminopyrimidin-6(1H)-one [33] had substrate activity, it decomposed too rapidly and the reproducibility of the initial-rate plots was very poor. The substrate concentrations in Table 5 are not true values because of the fast degradation of the quinonoid species. In the case of pterins the rates of rearrangement were reasonably slow, so one could use the concentration of tetrahydropterins as equal to the true concentrations of the quinonoid substrates. When app.K_m and app.V_{max} values were calculated in this experiment, the concentrations of the tetrahydropteridinones were taken as being the same as the quinonoid substrate concentrations. The true quinonoid substrate concentrations could be much smaller, so the true K_m values for these substrates will be smaller than the values in Table 5. The app.K_m values in phosphate buffer are slightly less than in Tris buffer and could reflect the higher proportion of quinonoid species

Table 5. Kinetic parameters of human brain dihydropteridine reductase for compounds, [15], [16] and [17] at 25°C.

7,8-Dihydro(6H)-pteridinones	app.K_m (μM)	app.V_{max} (u*/mg)	V/K	NADH (μM)
A				
Unsubstituted [15]	669(±2)	234(±0.4)	0.35	117
6-Methyl- [16]	366(±20)	131(±3)	0.36	117
6,7-Dimethyl- [17]	754(±45)	76(±4)	0.10	117
B				
Unsubstituted [15]	597(±20)	318(±8)	0.53	117
6-Methyl- [16]	276(±8)	444(±7)	1.61	80
6,7-Dimethyl- [17]	640(±52)	489(±27)	0.76	131

A In 0.1 M Tris/HCl buffer, pH 7.4

B In 0.3 M potassium phosphate buffer, pH 7.1

* μM NADH oxidized per min

(slower decomposition) in the former buffer. The app.V_{max} in Tris/HCl buffer for the respective quinonoid species, which were derived from compounds [18], [20] and [22], were in the order, [15] > [16] > [17]. Since these are the velocities at saturating concentrations of the quinonoid substrates, they are more meaningful constants than the app.K_m values. The values in phosphate buffer are higher than in Tris/HCl buffer but they are of the same order of magnitude.

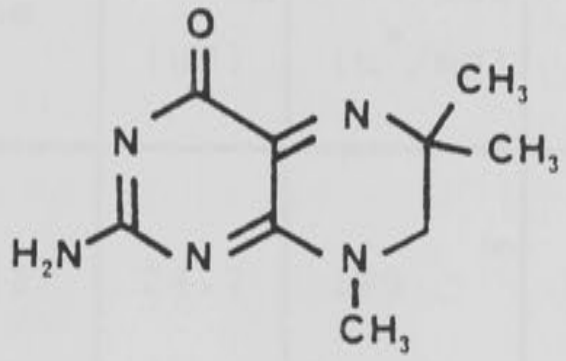
The reproducibility of the initial rates and the satisfactory spread of errors in the kinetic analyses confirm that the proportional concentrations of quinonoid species with respect to tetrahydropteridines used, at various concentrations of the latter, are the same within experimental error in each set of experiments.

Some of the substrates contained small amounts of borate salts and there was some concern that borate might inhibit the activity of the enzyme. No inhibition of DHPR, however, was observed at borate concentrations up to 1 mM when 6-methyl-7,8-dihydro(6H)pterin and NADH were used. The highest concentration of borate from the substrates in the assays was 0.9 mM, and most of the solutions contained considerably less than this concentration.

Kinetic parameters for compounds [24], [25], [28] and [30].

The kinetic parameters for the substituted pteridines are listed in Table 6. The quinonoid species

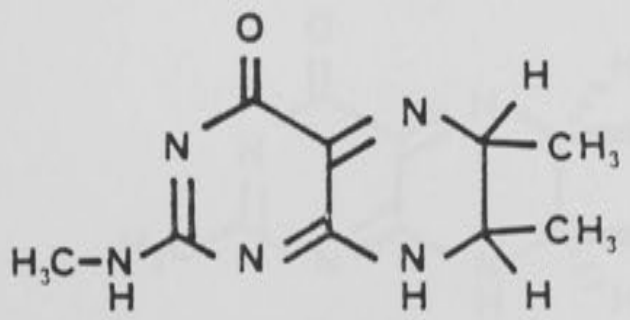
derived from compounds [24], [25], [26], [28], [29] and [30] were substrates for human brain DHPR. 6,6,8-Trimethyl 7,8-dihydro(6H)pterin [59] which is the oxidation product of compound [31] was not a substrate for human brain DHPR but was shown to be a weak inhibitor for the same enzyme by



[59]

Randles and Armarego.[126] The parameters of the known substrate 6,7-dimethyl-7,8-dihydro(6H)pterin [3] (p 92), were determined under identical conditions for comparison. In Tables 5 (p 128) and 6, the V/K values are a better indicators of the overall efficiency of the enzyme with a particular substrate than the separate app.K_m and app.V_{max} values, and the larger V/K value is an indication of better substrate efficiency.

Kaufman[68] had previously demonstrated that the quinonoid pterin [60] derived from the methylamino compound [25] (p 96) was a substrate by measuring only one initial



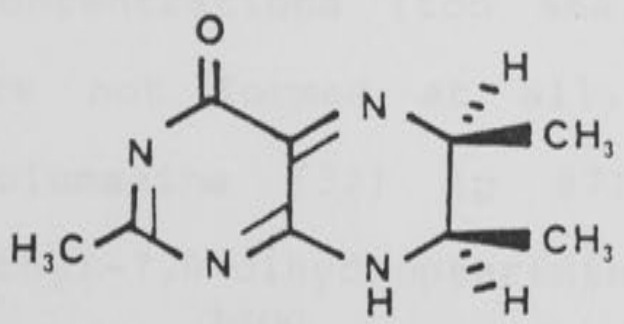
[60]

Table 6. Kinetic parameters of human brain dihydropteridine reductase for compounds [3], [57], [58], [59], [61] and [64] at 25°C.

7,8-dihydropteridin-4(6H)-one	app.K_m (μM)	app.V_{max} (u*/mg)	V/K	NADH (μM)	$K_3Fe(CN)_6$ (μM)
2-Amino-6,7-dimethyl- [3]	24.7	199	8	95	200
2-Amino-6,8-dimethyl- [64]	349	24.3	0.1	108	200
2-Methylamino-6,7-dimethyl- [60]	127	150	1	117	300
2-Methylthio-6,7-dimethyl- [57]	119.7	437.4	3.7	106	200
2-Methylthio-[6,7-D_2]-6,7-dimethyl- [58]	28.0	107.9	3.9	106	200
2,6,7-Trimethyl-[61]	38.8	10.1	0.3	111	360
NADH#	17.3	91.8			

* μM NADH oxidized per min

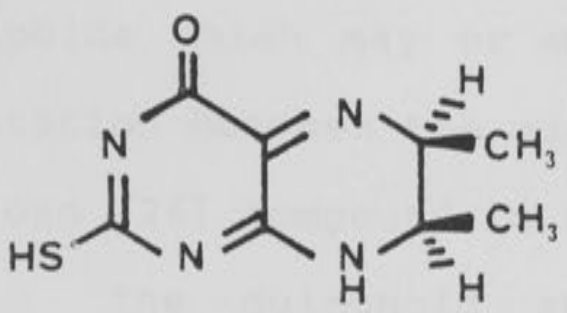
\# The concentration of 6-MeTHP as a substrate precursor was 50 μM.



[61]

rate. This rate was 36% of the initial rate of the quinonoid species [3] (p 92) derived from 6,7-dimethyl-5,6,7,8-tetrahydropterin [52] (p 113) when using the sheep liver DHPR. In the present work, the kinetic parameters for human brain DHPR were determined. The V/K values indicate that the enzyme activity with the pterin [60] as substrate was 12.5% of the activity when [3] was substrate.

The thioxopteridinone [27] (p 96) was oxidized by peroxidase and hydrogen peroxide or potassium ferricyanide as observed by the UV spectral changes. However, no DHPR active species could be detected in the oxidation products, and the TLC and UV spectral properties of the final oxidation product were similar to those of the original 6,7-dimethyl-2-thioxo-2,3-dihydropterin-4(1H)-one [46] (p 106) (see Section 3-6-8, p 182). This demonstrates that either the quinonoid species [62] were formed in very small



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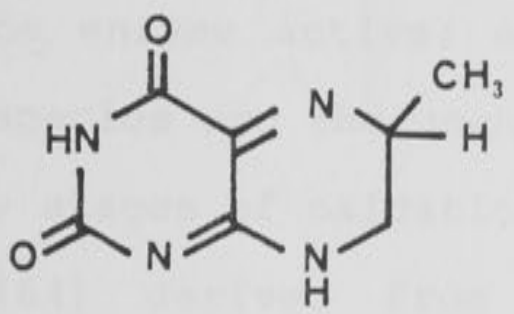


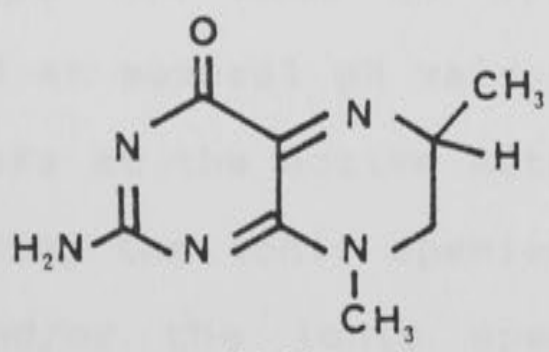
[63]

(steady state) concentrations (too small to show enzyme activity) or were not formed at all. Also 6-methyl-5,6,7,8-tetrahydrolumazine [32] (p 97) which had been oxidized to 6-methyl-7,8-dihydropteridin-2(3H),4(6H)-dione [63] (^{1}H NMR evidence),[73(b)] was found in the present work

not to be a substrate or inhibitor of human brain DHPR.

The oxidation product of 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26] (p 96) was found to be a substrate of human brain DHPR in this work, because this quinonoid species showed that i) maximum enzyme activity (i.e. initial rates of oxidation of NADH) was observed at least immediately after addition of enzyme, ii) at set concentrations of substrate and NADH, enzyme activity increased linearly with increase in enzyme concentration, iii) at constant enzyme and NADH concentrations but varying substrate concentrations the initial rates were hyperbolic, i.e. indicative of saturation kinetics. The kinetic parameters could not be obtained for this substrate, however, because it was not possible to reproduce the initial rate traces to within the accepted standard deviation. This is possibly due to oxidation of the 4-thio group to give the respective disulphide which may or may not be enzyme active; and/or dismutation between the quinonoid species and the unchanged 4-thioxo [26] compound in the early stages of oxidation.

The quinonoid species [64] derived from 6,8-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [30] by



[64]

oxidation proved to have substrate activity, although it is among the poorest of the active pteridines that were studied, and satisfied the above three general criteria for substrate activity (see Table 6, p 131).

The quinonoid species from the methylthio derivatives [28] and [29] showed higher efficiency as substrates than the quinonoid species from the methylamino derivative, although they decomposed more rapidly and the initial rates were more difficult to obtain experimentally because of the relative instability of the substrate. This indicates that the electrostatic effect of the substituent at the C-2 position (-SMe versus -NHMe) may play an effective role on the binding of the substrate to the enzyme.

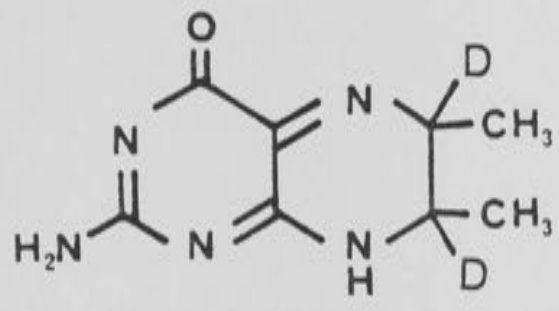
The K_m and V_{max} values of NADH for human brain DHPR were also measured by using quinonoid 6,7-dimethyl-7,8(6H)-dihydropterin [3] (p 92) as a substrate and gave similar values as those which were obtained for other sources of DHPR[22] (see Table 6, p 131).

3-3-5 pH rate profile study

An attempt was made to evaluate the kinetic parameters of DHPR at several pH values in order to obtain some idea of the pKa at the active site. This may provide information regarding the ionic species at the active site of the enzyme and/or the ionic species of the active substrate.

In this experiment, the quinonoid species were formed by oxidation with peroxidase and hydrogen peroxide in Tris/HCl buffer at pH ~7 and kept in an ice bath, because the rates of formation of quinonoid species varied with pH. The quinonoid species were stable at 0^0C (pH ~7) for 30 min. Before the assay, aliquots were taken and mixed with the mixed buffer (MES and ethanolamine)[145] to give final pH values between 5.7 ~ 9.8.

Quinonoid 6,7-dimethyl-[6,7-D_2]-7,8-dihydro(6D)-



[65]

pterin [65] was chosen as the substrate because it was known to rearrange very slowly ($t_{1/2}$ 112 min in Tris/HCl buffer, pH 7.6).[72] The pH rate profile for the rearrangement of this quinonoid species is shown in Figure 14. However, although a considerable number of kinetic runs were performed for DHPR activity, it was not possible to obtain values of the app.K_m and app.V_{max} within acceptable limits of error and reproducibility at pH values below 6 and above 10. Between pH 6 and 10 the enzymic rates were uniformly constant within experimental error. It was clear that below pH 6 the non-enzymic oxidation of NADH (Figure 15) varied with pH. Also the rates of rearrangement of

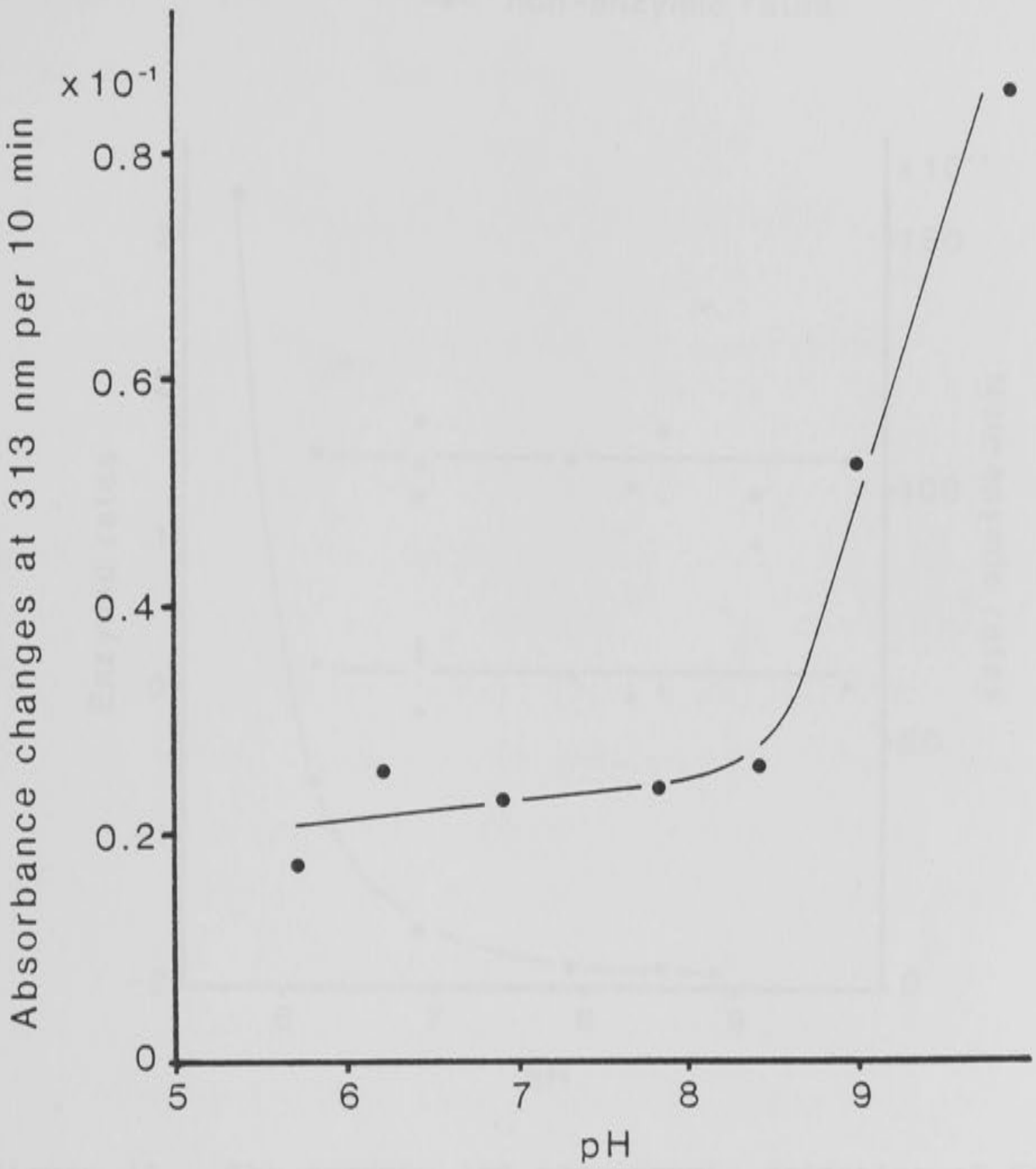


Figure 14. The stability of the quinonoid 6,7-dimethyl-[6,7-D_2]-7,8-dihydro(6D)pterin at different pH values.

The rates were measured at 310 nm, 28°C.

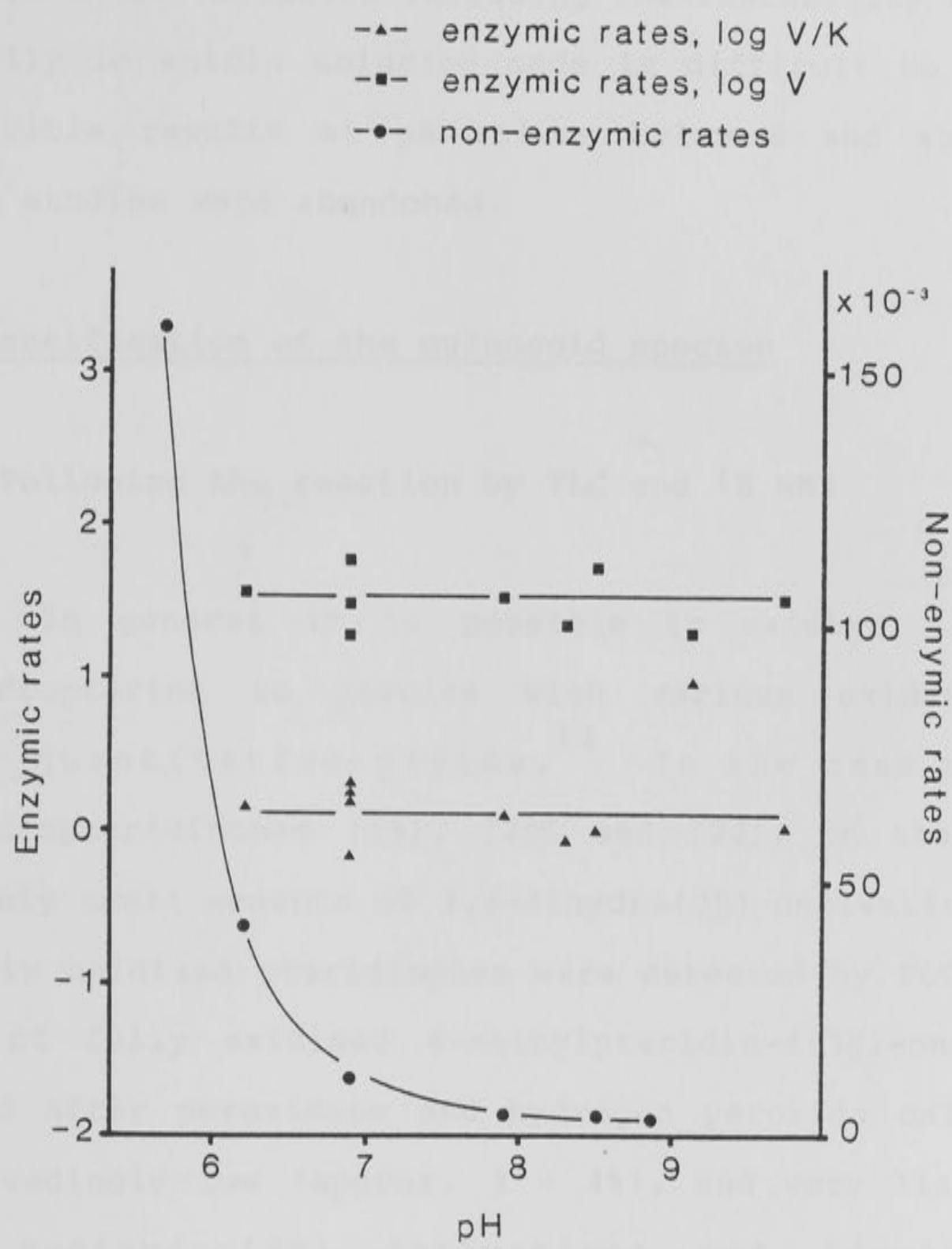


Figure 15. The enzymic and non-enzymic oxidation of NADH at different pH values. -▲- enzymic rates, log V/K; -■- enzymic rates, log V; -●- non-enzymic rates, v_0 (µmoles NADH oxidized/min/ml).

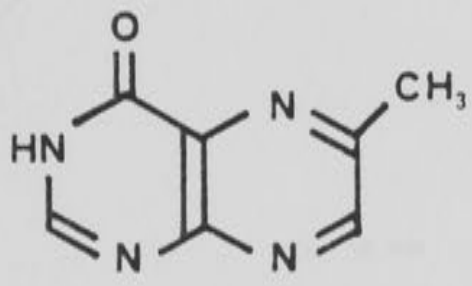
substrate to 7,8-dihydro(3H)pterin varied with pH (Figure 14). All these variables including the instability of NADH especially in acidic solution made it difficult to obtain reproducible results at pH values below 6 and above 9. Further studies were abandoned.

3-4 Identification of the quinonoid species

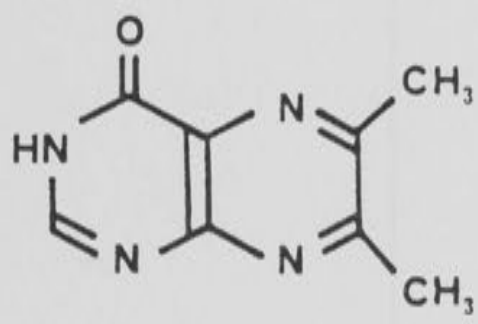
3-4-1 Following the reaction by TLC and ^{1}H NMR

In general it is possible to oxidize 5,6,7,8-tetrahydropterins to pterins with various oxidants in almost quantitative yields.[22] In the case of the tetrahydropteridinones [18], [20] and [22], on the other hand, only small amounts of 7,8-dihydro(3H) derivatives and the fully oxidized pteridinones were detected by TLC. The yields of fully oxidized 6-methylpteridin-4(3H)-one [37] isolated after peroxidase and hydrogen peroxide oxidation are exceedingly low (approx. 3 ~ 4%), and very little of the 7,8-dihydro(3H) derivatives was identified. Examination of the products of peroxidase and hydrogen peroxide oxidation of compounds [18], [20], [22] and [33] by removing samples at time intervals and separating the components by TLC showed that within a minute of mixing at least six spots appeared. Their relative intensities varied with time but the number of spots did not change with time. The reaction was repeated on a large scale for compounds [20] and [22], allowed to proceed for 10 h and

the products were separated by TLC. One band in each case was identified by UV spectra (at three different pH values), by ^{1}H NMR spectra and by TLC (re-run) as the respective fully oxidized compounds [37] and [36]. Most of



[37]



[36]

the other bands gave substances, in small amounts, that had no UV absorption and a few unidentified proton signals in the ^{1}H NMR spectra. The lack of UV absorption of some of the bands is indication of ring cleavage occurring with loss of chromophoric properties by hydration across the C2,N1 double bond. In the case of pyrimidine [33] (p 97) similar results were obtained (i.e. several spots on TLC), but 2-carboxy-1,3,5-triazin-4(3H)-one [66] or 1,3,5-triazin-2,4(1H,3H)-dione [67] were not identified among the products. These should have been the possible products if the oxidation proceeded as reported for 2,4,5-triamino-pyrimidin-6(1H)-one [54] (p 114).[146] However, these reported reactions did not involve peroxidase (cf. reference 147), i.e. the pyrimidinone [33] was reacted with hydrogen peroxide in water or hydrogen peroxide in alkaline solution. Similar oxidations were carried out in NMR tubes and the proton spectra were examined at time intervals (Figure 16). After 16 min, the downfield signal from the

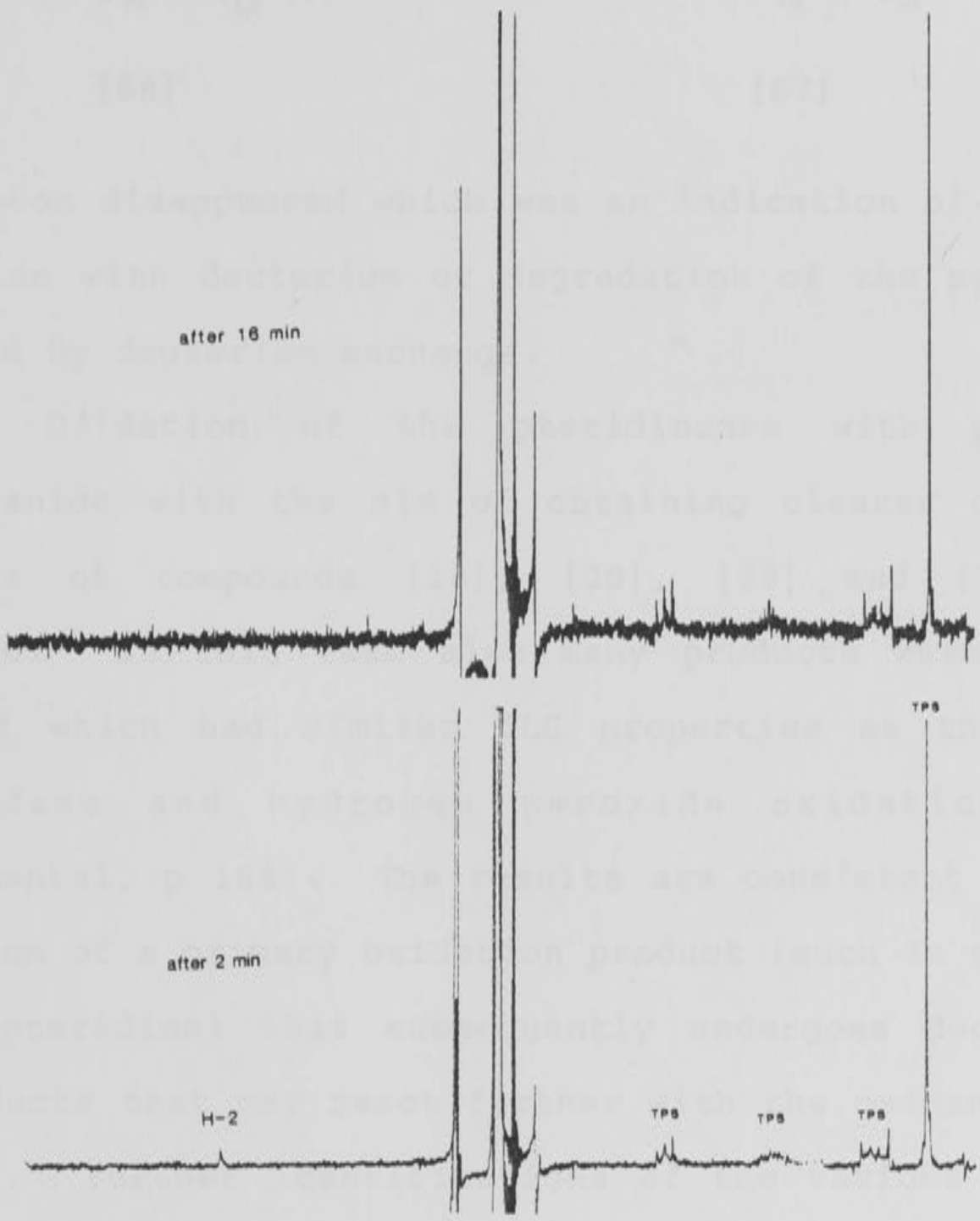
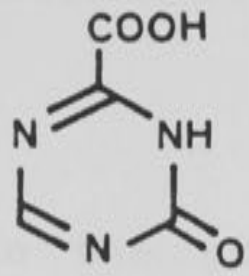
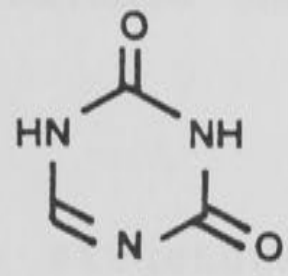


Figure 16. ^{1}H NMR spectrum of oxidation of 5,6-diamino-pyrimidin-4(3H)-one [33] by peroxidase and hydrogen peroxide.



[66]



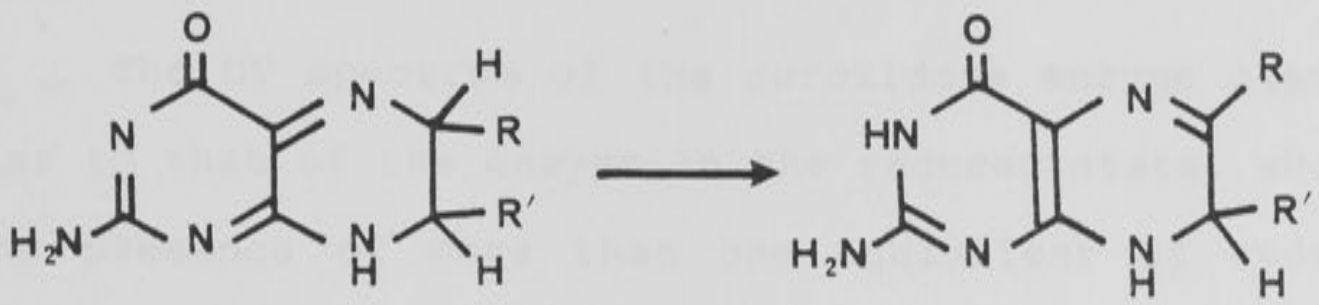
[67]

C-2 proton disappeared which was an indication of exchange of proton with deuterium or degradation of the pyrimidine followed by deuterium exchange.

Oxidation of the pteridinones with potassium ferricyanide with the aim of obtaining clearer oxidation products of compounds [18], [20], [22] and [33] were examined. In this case also many products were formed, some of which had similar TLC properties as those from peroxidase and hydrogen peroxide oxidations (see Experimental, p 186). The results are consistent with the formation of a primary oxidation product (such as quinonoid dihydropteridine) that subsequently undergoes degradation to products that may react further with the oxidant or the solvent. Further identifications of the various reaction products were frustrated by the small amounts produced and by the lack of definitive information revealed by their UV, IR and ^{1}H NMR spectra.

3-4-2 **Reaction with Horseradish peroxidase and hydrogen peroxide**

In the pterin series, the structures of the quinonoid species (which are substrates for DHPR) formed from the peroxidase and hydrogen peroxide oxidation of 5,6,7,8-tetrahydropterins, are well established.[22] Although these species rearrange to the corresponding 7,8-dihydro(3H)pterins (Scheme 7), they are stable enough for

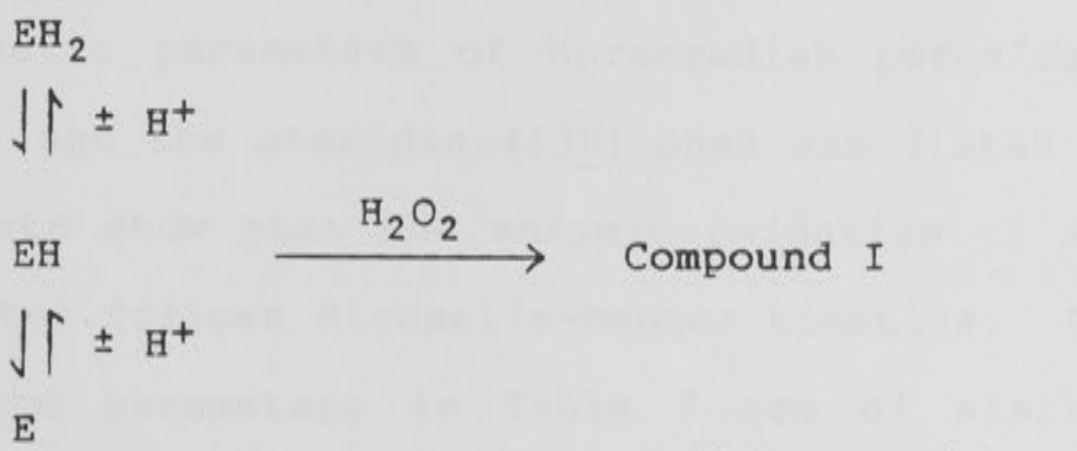


Scheme 7

some structural investigations. They can be generated so rapidly (e.g. within 20 sec in assay conditions) that intermediates cannot be detected under the present assay procedures. The pteridin-4(3H)-ones [18], [20] and [22] are also oxidized with peroxidase alone (or in buffer saturated with O_2), but very slowly (less than 0.1% in 10 min) compared with the oxidation of the pterins (e.g. 6-methyl- and 6,7-dimethyl-5,6,7,8-tetrahydropterins). The main difference in behaviour between the pterins and the pteridin-4(3H)-ones is that the quinonoid species from the latter are less stable and the quinonoid species are not

observed by UV spectroscopy. If the quinonoid species from the pterins and the pteridin-4(3H)-ones are both substrates for DHPR, they should give the same initial structures after oxidation and the differences in behaviour are due to the differences in oxidation potential between the two species (i.e. with and without the 2-amino group) and the following reaction pathways. In order to support this idea the substrate activities of both series of compounds were examined using the peroxidase and hydrogen peroxide system to see whether they exhibited clear Michaelis-Menten kinetics.

The UV spectrum of the peroxidase enzyme used was similar to that of the enzyme in the reduced state, whereas in the presence of more than one equivalent of hydrogen peroxide it was converted into Compound I which was the primary peroxide derivative of horseradish peroxidase (Scheme 8),[148] a process that was known to be very fast



Scheme 8

(second-order rate constant 0.9×10^7 $M^{-1}.s^{-1}$) and followed first order kinetics.[148,149] Like the pterins, the pteridin-4(3H)-ones showed saturation kinetics, i.e. the

rates of oxidation increased with increasing concentration of substrate, with a constant amount of peroxidase and hydrogen peroxide, until they reached maximum values.

In this study, the rate of oxidation of substrates was measured with various amounts of peroxidase and hydrogen peroxide and keeping the concentration of one of these two components constant while altering that of the other. Figure 17 illustrates the rates of oxidation of 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [22] which is typical. The rates increased linearly up to a concentration of peroxidase (Sigma type I) of 8 µg/ml. The decrease in rates at high hydrogen peroxide concentrations is probably due to the oxidation of the enzyme by the large amounts of hydrogen peroxide. For measuring the kinetic parameters, low concentrations of peroxidase were chosen from the linear portions of the peroxidase concentrations versus the initial velocities plots.

The kinetic parameters of horseradish peroxidase with the pterins and the pteridin-4(3H)-ones are listed in Table 7. The data show that the enzymic oxidation of all of these substrates follows Michaelis-Menten kinetics. The orders of all the parameters in Table 7 are of similar magnitude, so probably the enzymic oxidation mechanism of tetrahydropteridinones is the same as those of tetrahydropterins. However, the subsequent changes after the formation of the initial quinonoid species, are different.

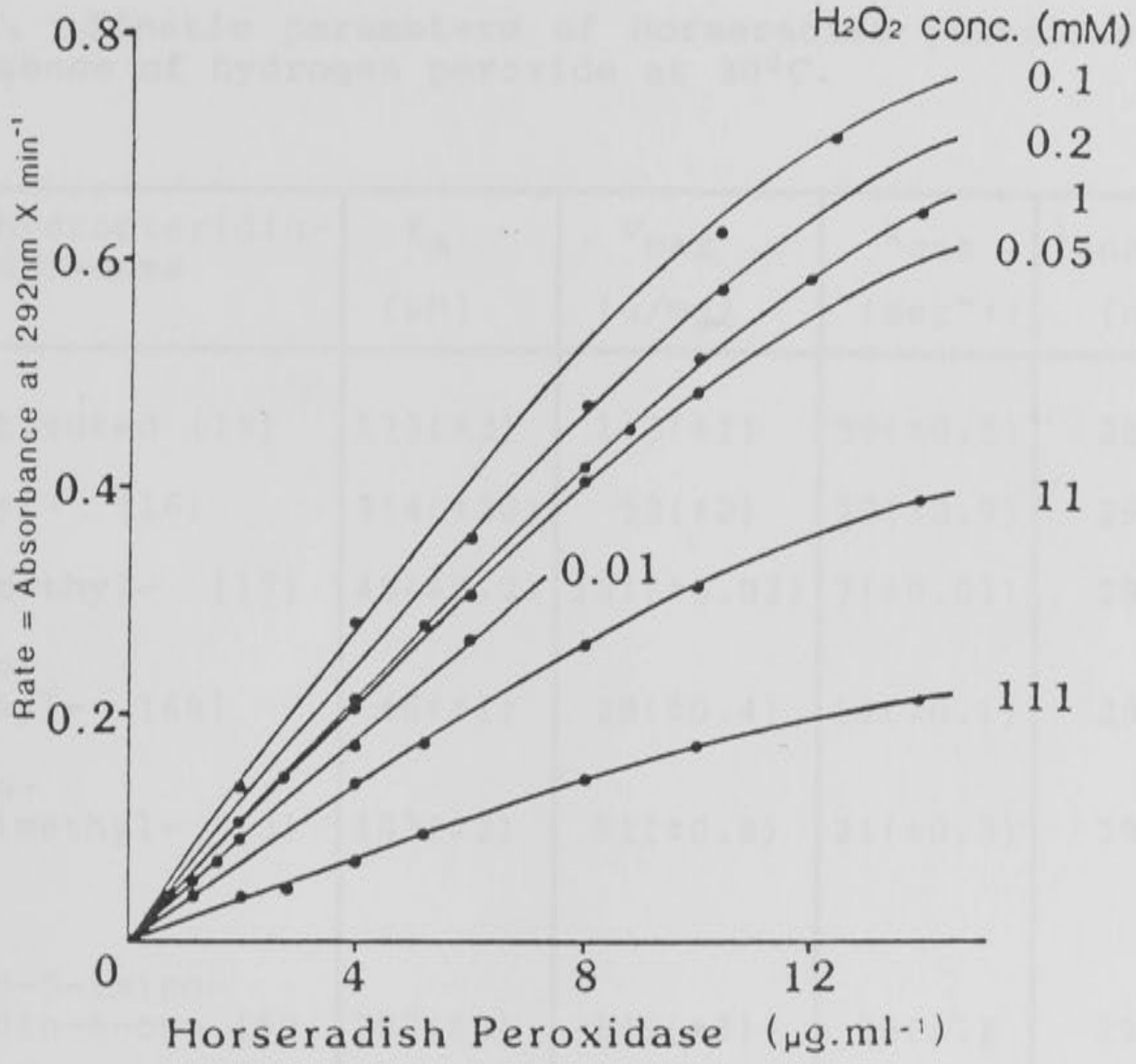
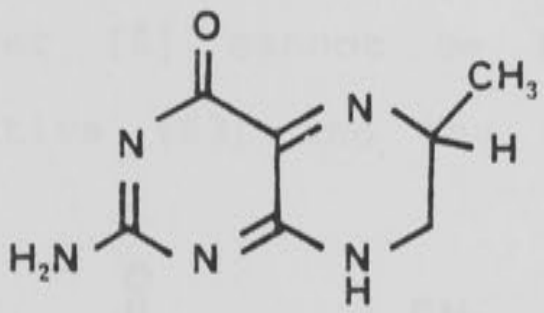


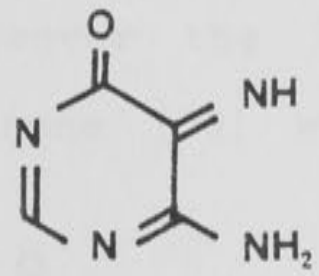
Figure 17. Effect of peroxidase (Sigma type I) and hydrogen peroxide concentrations on the initial rates of oxidation of 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [22] (at the concentrations shown) in 0.1 M Tris/HCl buffer (pH 7.4) at 25°C.

Table 7. Kinetic parameters of Horseradish peroxidase in the presence of hydrogen peroxide at 30^0C.

7,8-dihydropteridin-4(6H)-ones	K_m (μM)	V_{max} (u/mg)	k_{cat} (sec^{-1})	λ_{analyt} (nm)
Unsubstituted [15]	173(±3)	143(±1)	59(±0.5)	289
6-Methyl- [16]	314(±20)	53(±2)	22(±0.9)	290
6,7-Dimethyl- [17]	45(±0.2)	161(±0.03)	7(±0.01)	292
2-Amino-6-methyl- [68]	46(±1)	28(±0.4)	12(±0.1)	298
2-Amino-6,7-dimethyl- [3]	103(±2)	51(±0.8)	21(±0.3)	298
4-Amino-5-imino-pyrimidin-6-one [69]	203(±8)	149(±3)	56(±1)	298



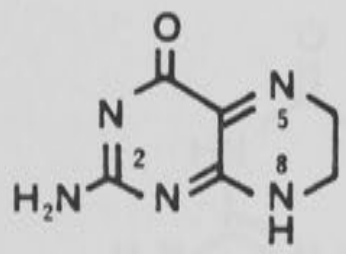
[68]



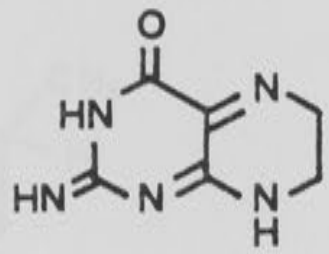
[69]

3-5 Conclusion

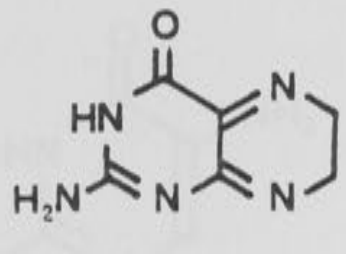
The 5,6,7,8-tetrahydropteridin-4(3H)-ones [18], [20] and [22] are effective substrates for horseradish peroxidase in the presence of hydrogen peroxide, similar to the related 5,6,7,8-tetrahydropterins. The transient quinonoid species derived from the oxidation of the tetrahydropteridin-4(3H)-ones are found to be substrates for human brain DHPR and the enzymic activities of the compounds which are used in this work strongly support the



[4]

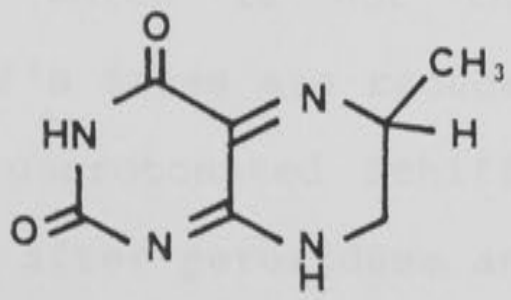


[5]

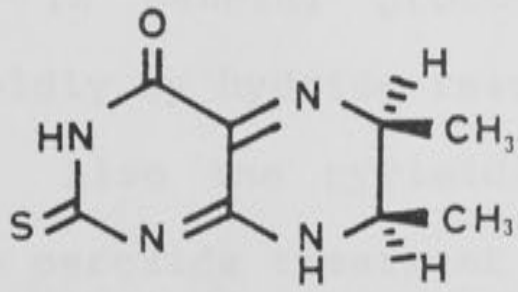


[6]

tautomeric structure [4] for the enzyme active species. When the C-2 position of the pyrimidine ring is unsubstituted, structure [5] cannot be formed. Also when it was substituted with methyl or thiomethyl groups, tautomer [5] cannot be formed. Moreover the lumazine derivative [63] and the thioxopteridinone [70] were not



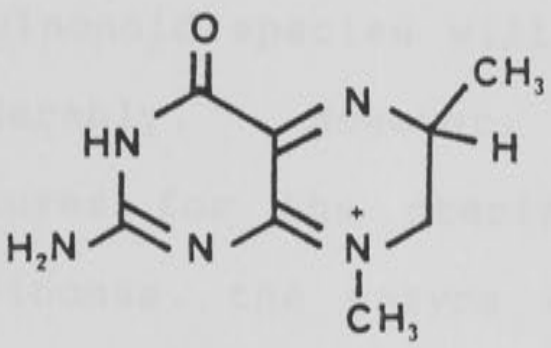
[63]



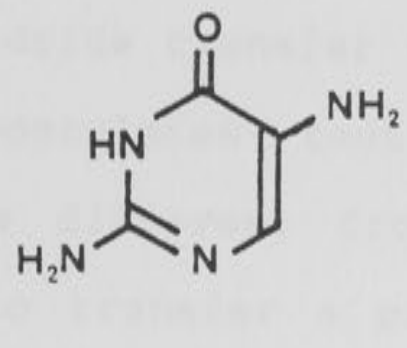
[70]

substrates for DHPR and support the exclusion of the

tautomeric structure [5]. However, compounds [18], [20], [22] and [33] could be oxidized to the ortho-quinonoid structure [6]. This tautomer was excluded because the 6,8-dimethyl compound [64] (p 133) was a substrate, albeit among the poorest studied. If tautomer [6] is formed in this case, the quinonoid structure of the 6,8-dimethyl compound [30] should give a cation with the charge on N-8 (i.e. [71]). This quinonoid species should deprotonate at pH ~7 (cf. pKa of 8-methylpterin 5.42,[150] 6,8-dimethyl-7,8-dihydropterin 4.40[151]) which will alter its tautomeric



[71]

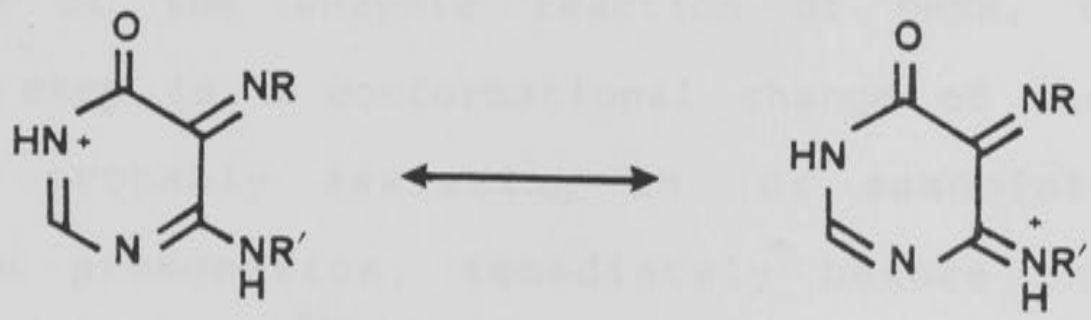


[72]

structure to [4]. The pKa of the quinonoid dihydropterin is ca 5.4[152,153] and at pH 7.4 it is almost entirely in the unprotonated form. If the substrate retained structure [71] then we should have expected an accelerated hydride transfer (in the absence of enzymic steric hindrance from 8-Me) which is not the case. In general protonated Schiff's bases are reduced more rapidly by hydride reagents than unprotonated Schiff's bases. Also the pyrimidinone [72], after peroxidase and hydrogen peroxide treatment (not studied in detail), exhibited some activity with DHPR.[48]

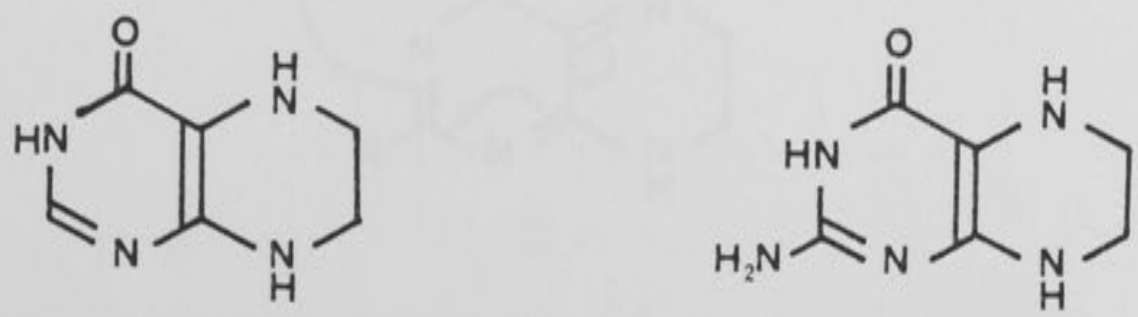
A structure such as [6] for the pteridin-4(3H)-

ones implies the remote possibility that DHPR can reduce two different tautomers, [4] and [6], of very similar substrates. It is true that the cations of the two tautomers are mesomeric, [73] and [74], and protonation of



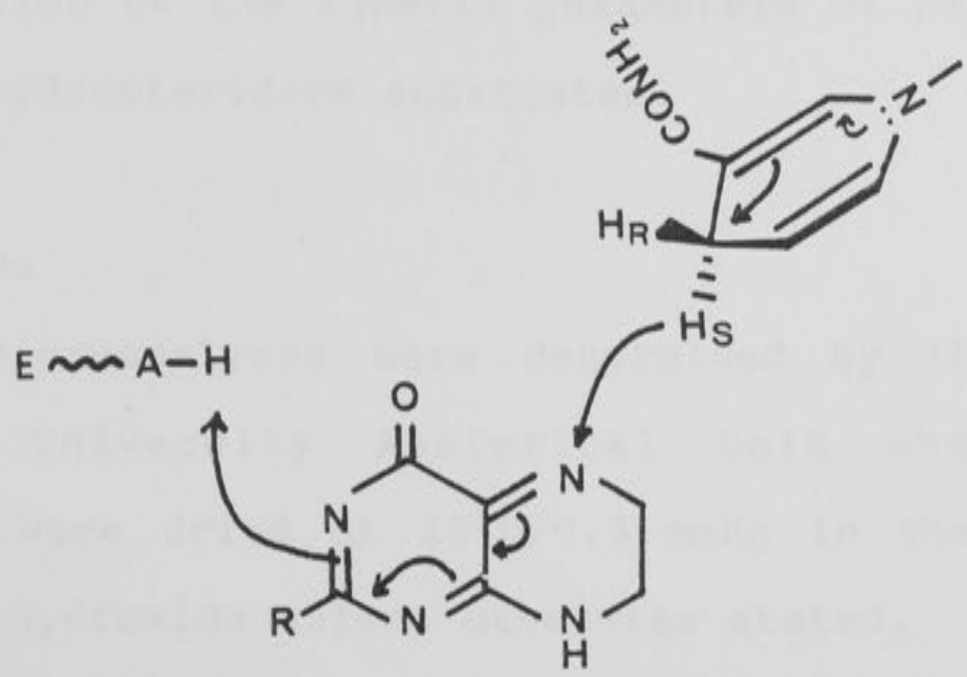
[73] [74]

the quinonoid species will enhance hydride transfer to N-5 considerably. However, if one postulates tautomeric structures for the pterins that are different from the pteridinones, the enzyme will need to transfer a proton, e.g., to N-3 in the case of the pterins and to N-8 in the case of the pteridinones after binding of the neutral species. The absence of a 2-amino group in the quinonoid dihydro(6H)pteridinone would be base-weakening when compared with quinonoid dihydro(6H)pterin and make its pKa lower than 5.4 by at least 1.6 pH units (cf. the pKa of 5,6,7,8-tetrahydropteridin-4(3H)-one [18] is 3.86[129] and 5,6,7,8-tetrahydropterin [75] is 5.6[139,140]). So these



[18] [75]

species are even less likely to be protonated at neutral pH. Protonation of the quinonoid species would most probably take place on the enzyme after or before hydride transfer, but this information is not known at the moment. However, according to Poddar and Henkin,[84] who studied the mechanism of the enzymic reaction of DHPR, the rate-limiting step is a conformational change of the ternary complex, probably assisting in (or associated with) substrate protonation, immediately before the faster hydride transfer (no deuterium isotope effect with NAD^2H) from NADH to the quinonoid substrate. This is followed by rapid release of products. So in the final conclusion the transfer of hydride to the quinonoid pterin is most probably the same as in the quinonoid pteridin-4(3H)-ones and that the tautomeric structure [4] is most probably the active form of the substrate. The chemical mechanism of the enzymic reaction can be summarized as in Scheme 9.



Scheme 9

3-6 Experimental

3-6-1 Materials and Methods

A: Materials

All chemicals were of the highest commercially available purity.

[1,2-D_2]-glyoxal bis-(sodium bisulphite) monohydrate[47] was kindly supplied by Professor H. Taguchi.

Horseradish peroxidase was from Boehringer grade II horseradish peroxidase (Boehringer, North Ryde, Australia) (A_{403}/A_{279} ratio 1.0) or Sigma type I horseradish peroxidase (Sigma, St. Louis, USA) (Rz 0.6, at double the concentration) and were used for the generation of quinonoid species in the DHPR assay. The former enzyme (found A_{402}/A_{272} ratio 1.35, ε_{403} 63 x 10^3 $M^{-1}.cm^{-1}$ in 1 mM phosphate buffer, pH 6.6, at 25^0C; lit.[154] values A_{403}/A_{275} ratio 3.25, ε_{403} 102 x 10^3 $M^{-1}.cm^{-1}$) was used for the determination of the kinetic parameters of peroxidase with the tetrahydropteridine substrates.

B: Methods

Microanalyses were determined by the Australian National University Analytical Unit and analytical specimens were dried at 25^0C/0.3 mmHg in the presence of potassium hydroxide unless otherwise stated.

^{1}H and ^{13}C NMR spectra were measured on a Jeol FX90Q spectrometer operating at 34^0C. Sodium 3-(trimethyl-

silyl)propionate (TPS) was used as internal standard for the ^1H NMR spectra (δ values are in ppm and $\underline{J}$ values are in Hz) in D_2O.

The EI mass spectra were measured with 16 eV ionizing electrons, 1.2 kV accelerating voltage on a Hitachi M-70 mass spectrometer (performed by Professor H. Taguchi, Kyoto Women's University, Japan). Samples were introduced by a direct insertion probe into the ion source maintained at 120^0C.

IR spectra (KBr disc) were measured on a Unicam SP 1050. The values in parentheses were shoulders.

Thin-layer chromatography (TLC) was run on silica gel 60 F_{254} (Merck, Hohenbrunn, FRG) and plates were eluted with n-butanol : acetic acid : water = 20 : 3 : 7. The spots were revealed by UV light (at 250 and 365 nm) and by staining with iodine vapour. In large scale experiments, each band was extracted with 1 : 1 methanol - methylene chloride.

Melting points were measured in open (pyrex) tubes.

UV spectra and kinetic measurements were performed as described before (see Chapter 2, Section 2-9-1, p 73).

The accurate absorbance for evaluating concentrations was performed on a Perkin-Elmer Lambda 1 single beam spectrometer.

Human brain DHPR used in the present chapter was purified by Mr Bela Paal and was homogeneous by SDS gel electrophoresis.

3-6-2 Syntheses

4,5-Diaminopyrimidin-6(1H)-one [33]: 4,5-Diaminopyrimidin-6(1H)-one was prepared as described[127] with slight modifications. Ethyl cyanoacetate (25 g, 0.25 mol) was dissolved in acetic acid (45 g, 0.75 mol) and then sodium nitrite (51.8 g, 98.5%, 0.75 mol) in water (71 ml) was added to it with cooling (below 10^0C) (cf. reference 155). The crystalline mass was extracted with ether (discard), washed with saturated NaCl solution and then aqueous sodium bicarbonate and dried. The crystals were triturated with benzene to give ethyl hydroxyiminocyanoacetate (29.2 g, 82.3%).

The ethyl hydroxyiminocyanoacetate (14.2 g, 0.1 mol) was condensed with thiourea (7.6 g, 0.1 mol) in sodium ethoxide solution [from sodium (4.6 g, 0.2 mol) and ethanol (250 ml)]. After boiling under reflux for 3 h, the brown-red sodium salt of 4-amino-2-mercapto-5-nitrosopyrimidin-6(1H)-one (quantitative) was filtered off, washed with a little ethanol and dried. To the cooled solution (18 ~ 35^0C) of the sodium salt (19.5 g, 0.1 mol) in 1 M NaOH (430 ml), sodium dithionite (68.9 g, 396 mmol) was added during 20 min with stirring. Further portions of sodium dithionite (18 g, 87.9 mmol and 6.5 g, 31.7 mmol) were added to the reaction mixture, because reduction was not complete (colour was not discharged), and stirring was continued for 15 min at room temperature (pH 6 ~ 7). Under cooling, acetic acid was added to give pH ~5. The pale

mercapto-diamine that separated (10.9 g, 63.8%) was filtered off, washed with a little water, ethanol and ether, and dried. The mercapto-diamine (26.6 g, 0.17 mol) was dissolved in 1.6% aqueous ammonia (539 ml), heated to boiling, and Raney nickel catalyst (76 g) was added in portions. This mixture was boiled under reflux for 2 h and filtered. The filtrate was evaporated to half its volume and cooled. The solid was filtered off, washed with boiling water and a little ethanol and dried to give 4,5-diaminopyrimidin-6(1H)-one (12.4 g); m.p. 238-242^{0}C (dec.) [lit.[127] m.p. 239^{0}C]. From the mother liquor, a further quantity [3.5 g, m.p. 238-243^{0}C (dec.)] was obtained. The total yield was 74% (lit.[127] 76%). (Found: C, 33.9; H, 5.6; N, 39.8%. $C_4H_6N_4O$, $0.8H_2O$ requires C, 34.2; H, 5.5; N, 39.3%); ^{1}H NMR (D_2O): δ 7.76, s, H2; UV : ε_{278} 8912 (pH 7).

Pteridin-4(3H)-one [35]: Pteridin-4(3H)-one was prepared as before[127] with slight modifications. To a solution of glyoxal bis-(sodium bisulphite) (3.12 g, 11.0 mmol) in water (39 ml), 4,5-diaminopyrimidin-6(1H)-one (1.3 g, 10.3 mmol) was added and boiled under reflux for 1 h. The pH was adjusted to 0.5 with 11 M HCl, boiled vigorously for 10 min and adjusted to pH 3.5 with 2.5 M NaOH and then cooled. The solution was evaporated to dryness, dissolved in 1% aqueous ammonia, filtered off and passed through an aluminium oxide (Merck, Aluminium oxide 90 active neutral;

46 g) column and eluted with 1% aqueous ammonia. The main fraction was evaporated to dryness and the residue was dissolved in hot water and boiled with a little charcoal and then filtered. The filtrate was adjusted to pH 3 ~ 4 and cooled. The solids were collected and dried. The aluminium oxide column chromatography was repeated and recrystallization of the product from hot water (70 ml) gave pteridin-4(3H)-one (929 mg, 61.1%) (very pale brown). This was recrystallized further from hot water and gave almost pure compound (540 mg). It was then sublimed at $270^0C/9 \times 10^{-1}$ mmHg (pale yellow) (Found: C, 49.0; H, 2.7; N, 38.1%. Calculated for $C_6H_4N_4O$: C, 48.8; H, 2.7; N, 37.8%); ^1H NMR (1 M DCl): δ 8.97, d, J 2.3, H6 or H7; 9.05, d, J 2.3, H7 or H6; 9.10, s, H2; (1 M NaOD): δ 8.46, s, H2; 8.74, d, J 2.2, H6 or H7; 8.85, d, J 2.2, H7 or H6 ; IR: ν_{max} 3030(3080), 2885, 1724, 1617, 1590, 1550(1560), 1468, 1398(1410), 1374, 1320, 1280, 1228(1220), 1163, 1108, 1032, 923(913), 872(882), 858, 827, 720(712) and 607 cm^{-1}.

5,6,7,8-Tetrahydropteridin-4(3H)-one [18]:

i) Catalytic reduction with Raney nickel[128]: A suspension of pteridin-4(3H)-one (225 mg, non-sublimed material, 1.52 mmol) in ethanol (20 ml) was added to Raney nickel (2.0 g) in ethanol (10 ml). The mixture was hydrogenated for 3 h (25^0C/720 mmHg). The catalyst was filtered through Celite and washed with a little ethanol.

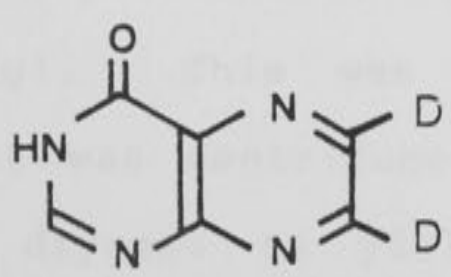
The filtrate was evaporated to dryness and the residue was dissolved in hot ethanol (1 ml), concentrated to half its volume and ether was added. The solid was collected (3 mg, 1.3%) and identified as 5,6,7,8-tetrahydropteridin-4(3H)-one (lit.[128] yield 60 %). This low yield may be due to an unknown impurity in the starting material.

ii) Attempted catalytic reduction with platinum oxide in trifluoroacetic acid: To a suspension of pre-reduced platinum oxide (25 mg) in trifluoroacetic acid (5 ml), a solution of pteridin-4(3H)-one (100 mg, 0.68 mmol) in trifluoroacetic acid (2.5 ml) was added, and the mixture was hydrogenated at 25^0C/720 mmHg. After 3.5 h, a further quantity of pre-reduced platinum oxide (50 mg) in trifluoroacetic acid (5 ml) was added and hydrogenation was continued for further 4 h. The UV spectrum in acidic solution (λ_{max} at 270 and 310 nm) did not show any further change indicating that reduction had not occurred to any appreciable extent. The catalyst was filtered off and 0.7 M methanolic-HCl (5 ml) was added to the filtrate followed by ether (60 ml) and methanolic-HCl (5 ml). The solid (~1 mg) that separated was washed with ether by centrifugation until the pH was neutral, and dried in vacuo. A broad signal appeared near 4 ppm in the ^{1}H NMR spectrum but there was no down field signal from the C-2 proton in DCl.

iii) Reduction with sodium borohydride: Sodium borohydride (511 mg, 13.5 mmol) was added to a solution of

pteridin-4(3H)-one (500 mg, 3.4 mmol) in 1 M aqueous sodium carbonate (4.7 ml). The reaction was followed by UV spectra and TLC properties. The reduction was not complete after 10 min, and the solution was heated for 1 min. After 20 min the reduction was still not complete and a further amount of sodium borohydride (100 mg) was added and heated for 5 min. After 35 and 55 min, further amounts of sodium borohydride (100 mg) were added and the mixture was heated for 5 min after each addition. Reduction was then complete as evidenced by UV spectra and TLC. The pH of the solution was adjusted to 8 ~ 9 with acetic acid. The solids were filtered (most of this was inorganic material), the filtrate was freeze-dried and the residue was extracted with cold ethyl acetate (40 ml x 5) and then cold ethanol (40 ml x 4). Both extracts were evaporated to dryness. A very small amount of solid (1.2 mg) was obtained from the ethyl acetate extract. The residue from the ethanol extraction was triturated with water (5 ml), concentrated to half its volume and the white crystals were collected. On drying, these became slightly yellow (not hygroscopic, 120 mg), m.p. >190^{0}C (dec.) [lit.[128] m.p. 230^{0}C (dec.) obtained by potassium borohydride reduction] (Found: C, 41.5; H, 5.7; N, 31.8%. $C_6H_8N_4O$, $0.1NaH_2BO_3$, H_2O requires C, 41.2; H, 5.6; N, 32.0%). The tetrahydropteridinone crystals from various preparation always contained small amounts of borate (green-edged flame test with H_2SO_4/ethanol) although they gave one spot on TLC and clean ^{1}H NMR spectra. ^{1}H NMR (1 M DCl): δ 3.69, m, H-6,6, 7,7 and 8.59, s, H2.

[6,7-D_2]-pteridin-4(3H)-one [73]: This deuteriated pteridinone was prepared as before[127] for the undeuteriated compound, with slight modifications. A mixture of 4,5-diaminopyrimidin-6(1H)-one (1.3 g, 10.3 mmol) and [1,2-D_2]-glyoxal bis-(sodium bisulphite) monohydrate[47] (3.15 g, 11.0 mmol, 99.5% deuteriated at C-1 and -2) in water (39 ml) was refluxed for 1.7 h. After cooling the pH was adjusted to 1 with 11 M HCl and the solution was boiled vigorously for ca 10 min, (the colour had changed to dark purple) and evaporated to dryness. The residue was dissolved in 1% aqueous ammonia (8 ml) and filtered. The filtrate was passed through an aluminium oxide column (46 g in 1% aqueous ammonia) and eluted with 1% aqueous ammonia. The main fraction was evaporated to dryness. The residue was recrystallized from water (24 ml) to give the pteridinone (320 mg). This was dissolved in 1% aqueous ammonia (4 ml) and passed through a second aluminium oxide column (12 g in 1% aqueous ammonia) and eluted with 1% aqueous ammonia. The main fraction was evaporated to dryness (282 mg) and recrystallized from water (8 ml) to give purer pteridinone (141 mg). More solid (56 mg) was obtained from the mother liquor. The total yield of pteridinone was 12.7%. Anhydrous [6,7-D_2]-pteridin-4(3H)-one [76] (33 mg),



[76]

m.p. >303^{0}C (dec.) was obtained by sublimation (49 mg) at 230^{0}C/1 mmHg [Found: C, 48.2; H+D, 4.2; N, 37.5%; m/e 149 (100%), 150 (30%). [This fragmentation was different from previous reports[156] which used 70 or 80 eV as ionizing electrons. The differences in the electron strengths may be the cause for different fragmentation.] $C_6H_2D_2N_4O$ requires C, 48.0; H+D, 4.2; N, 37.3%; M^+ 150, IR : ν_{max} 3480, 3090(3060), 2925, 1724(1708), 1615(1600), 1545, 1434, 1378, 1322, 1268(1280), 1210, 1182, 1150, 1013, 1000, 990, 925(883), 836(823), 710 and 652 cm^{-1} (compare with non-deuteriated pteridin-4(3H)-one, p 155)].

[6,6,7,7-D_4]-5,6,7,8-tetrahydropteridin-4(3H)-one [19]: To a solution of [6,7-D_2]-pteridin-4(3H)-one (80 mg, 0.53 mmol) in 1 M sodium carbonate (in D_2O, 0.8 ml), sodium borodeuteride (Aldrich, Milwaukee, USA) (80 mg) was added and nitrogen gas was blown over the surface and capped tightly. The reaction was followed by UV spectra and TLC. This reaction mixture was treated with sodium borodeuteride (40 mg, each time) and heated for 30 sec after 20, 75, 180, 250 and 395 min. After 8 h, the pH was adjusted to 8 ~ 9 with 11 M HCl and cooled. The solid was collected and triturated with small portions of water to give the crude pteridinone (423 mg). This was extracted with ethyl acetate, the extract was centrifuged and the supernatant was evaporated to dryness to yield the tetra-deuterio pteridinone (23 mg) (Found: C, 41.4; H+D, 8.55; N, 31.5%.

Calculated for $C_6H_4D_4N_4O$, $0.02NaH_2BO_3$, H_2O: C, 41.0; H+D, 8.1; N, 31.9%); [Found: m/e 156 (100%), $C_6H_4D_4N_4O$ requires M^+ 156].

6-Methyl-5,6,7,8-tetrahydropteridin-4(3H)-one [20]: 6-Methylpteridin-4(3H)-one (2 g, which was kindly supplied by Dr D.J. Brown) was recrystallized from water (90 ml) (1.73 g, 86.5%; orange). The mother liquor was concentrated to 1/4 its volume and cooled (0.13 g, 6.5%; white). ^{1}H NMR showed that the crystals were pure 6-methylpteridin-4(3H)-one.[129]

i) **Catalytic reduction with platinum oxide in methanol:** Recrystallized 6-methylpteridin-4(3H)-one (150 mg) in methanol (300 ml) and platinum oxide (250 mg, pre-reduced in 10 ml of methanol) were shaken with hydrogen (25^{0}C/720 mmHg) for 4.2 h. A further quantity of platinum oxide (100 mg, pre-reduced in 10 ml of methanol) was added and shaking was continued for a further 1.5 h. The solution was filtered and evaporated to dryness. The residue (144 mg) was recrystallized from water (10 parts) to give 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one (30.2 mg), m.p. >211^{0}C (dec.). The crystals were pure by TLC and had UV spectra as reported[128] [ε_{290} 8511 (pH 7), ε_{259} 7079 (pH 1)] (Found: C, 45.9; H, 6.3; N, 29.9%. $C_7H_{10}H_4O$, H_2O requires C, 45.65; H, 6.6; N, 30.4%).

ii) Reduction with sodium borohydride: The reaction was carried out as before[128] with slight modifications. To a solution of 6-methylpteridin-4(3H)-one (500 mg, 3.09 mmol) in 1 M sodium carbonate (3 ml), sodium borohydride (500 mg, 13.2 mmol) was added during 3 min. An hour later the solution was warmed (effervescence) and after 1.75 h, the pH of the solution was adjusted to 8 ~ 9 and cooled. The solid was collected and washed with small portions of water. The product was recrystallized from hot water (2 ml) under nitrogen gas. The solid was collected and washed with a small portion of cold water to give 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one (349 mg, 68.0%), m.p. >200^{0}C (dec.). This solid could not be freed from small amounts of borate (flame test) by recrystallization (Found: C, 36.4; H, 5.4; N, 24.0%. $C_7H_{10}N_4O$, $0.76NaH_2BO_3$, $0.1H_2O$ requires C, 36.3; H, 5.1; N, 24.2%.); ^{1}H NMR (1 M DCl): δ 1.48, d, J 6.3, 6-Me; 3.39, q, J_{gem} -14.4, J_{vic} 9.4, $H7_{ax}$; ~3.7, m, H6; 3.77, q, J_{gem}-14.4, J_{vic} 3.0, $H7_{eq}$; 8.2, s, H2. [lit.[128] m.p. 217-218^{0}C (sealed tube); prepared in 60% yield with potassium borohydride.]

6-Methyl-2,3,5,6,7,8-hexahydropteridin-4(3H)-one [38]: To pre-reduced platinum oxide (50 mg) in trifluoroacetic acid (2.5 ml), 6-methylpteridin-4(3H)-one (200 mg) in trifluoroacetic acid (5 ml) was added and this suspension was shaken with hydrogen (25^{0}C/720 mmHg). After 2 h, the

catalyst was filtered off, the filtrate was added to an ice-cold solution of 1.3 M methanolic-HCl (2 ml) followed by dilution with dry ether (60 ml), whereby the colourless hydrochloride separated. This was collected and washed with ether (15 ml x 3) by centrifugation and then dried *in vacuo*. These crude solids (242 mg) were further purified from methanol containing a few drops of methanolic-HCl and excess ether, and had m.p. 213-218^{0}C (effervescence); (Found: C, 35.5; H, 6.1; Cl, 21.6; N, 23.1%. $C_7H_{12}N_4O$, 1.45HCl, H_2O requires C, 35.2; H, 6.5; Cl, 21.5; N, 23.4%); UV: ε_{258}(inflex) 158 (pH 0), ε_{293} 1054 (pH 7.3) and ε_{286} 1776 (pH 13.2); ^{1}H NMR (1 M DCl): δ 1.62, d, *J* 6.2, 6-Me; 3.61, q, J_{gem} -14.2, J_{vic} 10.6, $H7_{ax}$; 3.95, q, J_{gem} -14.2, J_{vic} 4.4, $H7_{eq}$; ~4.0, m, H6; 4.99, s, H2 (see Figure 1, p 102); ^{13}C NMR (proton coupled) (D_2O, dioxan as an internal standard 67.6 ppm downfield from tetramethylsilane): δ 16.0, q, *J* 131, 6-Me; 44.7, m, C7; 49.2, m, C6; 52.1, t, *J* 159, C2; 157.6, s, C8a; 162.9, s, C4 (see Figure 2, p 103).

6-Methyl-[6,7-D_2]-5,6,7,8-tetrahydropteridin-4(3*H*)-one [21]: To a solution of 1 M sodium carbonate (1 ml), 6-methylpteridin-4(3*H*)-one (100 mg) was added followed by sodium borodeuteride (100 mg). The reaction was carried out under a stream of nitrogen, and the solution was refluxed for 30 sec after 28, 55 and 100 min. After 3.3 and 4 h, further quantities of sodium borodeuteride (50 mg

each) were added and heated until clear. After 5 h, the pH of the solution was adjusted to 8 ~ 9 with 11 M HCl and cooled. The solid that separated was collected, triturated with water and dried. The solid (234 mg) was recrystallized from water to yield the required tetrahydropteridin-4(3H)-one (75 mg), m.p. 213-218^{0}C (dec.), which still contained borate (flame test) (Found: C, 44.5; H+D, 7.3; N, 29.3%. Calculated for $C_7H_8D_2N_4O$, $0.1NaH_2BO_3$, $0.9H_2O$: C, 44.0; H+D, 7.3; N, 29.5%), [Found: m/e 168 (100%), 153 (75%), 139.3 (metastable). $C_7H_8D_2N_4O$ requires M^+ 168, M^+-15(Me) 153]. The UV spectra (ε_{290} 9685, pH 7.0; lit.[128] for 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one ε_{290} 8511, pH 7) and TLC properties were identical with those of 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one above. The deuterium incoporation was better than 95% (from ^{1}H NMR spectra by comparison with CH_3 integral).

6,7-Dimethylpteridin-4(3H)-one [36]: The reaction was carried out as before[127] with slight modifications. A solution of 4,5-diaminopyrimidin-6(1H)-one (2.0 g, 15.9 mmol) in ethanol (200 ml) and water (20 ml) was heated to boiling and diacetyl (2 ml) was added to it. The solution was boiled under reflux for 1 h and cooled. The solution was concentrated to half its volume and cooled at 4^{0}C. The precipitate (2.42 g) was collected, washed with a little, cold ethanol and ether, and dried in vacuo. The mother liquor was concentrated to half its volume, cooled and the

solid was collected, washed and dried to give a further quantity of pteridine (64 mg). The total yield was 87.9%; ^{1}H NMR (1 M DCl): δ 2.78, s, 7- or 6-Me; 2.81, s, 6- or 7-Me; 9.23, s, H2.

cis-6,7-Dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [22]: The preceeding 6,7-dimethylpteridin-4(3H)-one (500 mg) in 3 M HCl (25 ml) was hydrogenated with platinum oxide (100 mg, pre-reduced in 25 ml of 3 M HCl) for 5.25 h. The catalyst was filtered off and the filtrate evaporated to dryness. The colourless residue was dissolved in hot ~3 M methanolic-HCl (40 ml). The crystals that separated on cooling were collected and dried *in vacuo* to give the hydrochloride of the title compound (500 mg); m.p. >219^{0}C (dec.) (Found: C, 36.7; H, 6.1; Cl, 25.5; N, 21.3%. Calculated for $C_8H_{12}N_4O$, 1.87HCl, 0.7H_2O: C, 36.7; H, 6.1; Cl, 25.5; N, 21.3%); ^{1}H NMR (0.5 M DCl): δ 1.28, d, *J* 6.6, 6- or 7-Me; 1.35, d, *J* 6.6, 7- or 6-Me; 3.85 and 3.93, m, *J* 6.6 and 3.3, H6 and H7; 8.09, s, H2 (see Figure 3, p 105); UV: ε_{262} 6680 (pH 0) and ε_{292} 9025 (pH 7.0).

cis-6,7-Dimethyl-[6,7-D_2]-5,6,7,8-tetrahydropteridin-4(3H)-one [23]: To a suspension of pre-reduced platinum oxide (50 mg) in 3 M DCl in D_2O (15 ml), a solution of 6,7-dimethylpteridin-4(3H)-one (250 mg, 1.42 mmol) in 3 M DCl (20 ml) was added, and this suspension was hydrogenated

with D_2 gas (25^{0}C/720 mmHg) for 6 h. The catalyst was removed by filtration through Celite and the filtrate was evaporated. The residue (crude, 0.45 g) was dried *in vacuo* over phosphorous pentoxide. The solid was dissolved in methanol (4 ml) and 7 M methanolic-HCl (5 ml) was added followed by ether until turbid. The solution was cooled and the solid was collected (centrifugation) to give *cis*-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one hydrochloride (241 mg, slightly yellow); m.p. >218^{0}C (dec.) (Found: C, 34.3; H+D, 6.6; Cl, 25.2; N, 19.7%. Calculated for $C_8H_{10}D_2N_4O$, 2HCl, 1.4H_2O: C, 34.3; H+D, 6.7; Cl, 25.3; N, 20.2%), [Found: m/e 184(87%), 182(38%), 167(100%), 152(75%), 138(55%), $C_8H_{10}D_2N_4O$ requires M^+ 182, M^++2(?) 184, M^+-15(Me) 167, M^+-30(2Me) 152, M^+-44(?) 138]. ^{1}H NMR integrals indicated better than 95% deuterium incorporation at C6 and C7.

2,6,7-Trimethylpteridin-4(3*H*)-one [45]: The reaction was carried out as before[157] with slight modifications. 4,5-Diamino-2-methylpyrimidin-6(1*H*)-one sulphate,[133] which was generously supplied by Dr D.J. Brown, (0.5 g) and diacetyl (5 ml) in water (10 ml) was boiled under reflux for 1.5 h. The pH was adjusted to 4 then cooled to give the 2,6,7-trimethylpteridin-4(3H)-one which had m.p. >241^{0}C (dec.) (lit.[157] m.p. 261-262^{0}C) (138 mg, 55 %), after washing with ethanol and drying (Found: C, 54.5; H, 5.3; N, 28.55%. $C_9H_{10}N_4O$ requires C, 54.8; H, 5.5; N, 28.4%);

^{1}H NMR (D_2O): δ 2.54, s, 2-Me; 2.66, s, 6- or 7-Me; 2.68, s, 7- or 6-Me.

cis-2,6,7-Trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one [24]: Pre-reduced platinum oxide (50 mg) in 3 M HCl (5 ml) and 2,6,7-trimethylpteridin-4(3H)-one (50 mg, 263 μmol) was shaken with hydrogen at 20^{0}C/720 mmHg for 9.5 h and the catalyst was filtered off. The filtrate was evaporated to dryness. The residual solid (70 mg) was dissolved in 7 M methanolic-HCl and ether was added until turbid and cooled. 2,6,7-Trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one hydrochloride was collected and dried (yield 30 mg, 10.3%). It had m.p. 210.5-211.5^{0}C (dec.) (Found: C, 37.2; H, 6.3; Cl, 23.7; N, 19.1%. Calculated for $C_9H_{14}N_4O$, 2HCl, 1.4H_2O : C, 37.1; H, 6.5; Cl, 23.9; N, 19.2%); ^{1}H NMR (D_2O): δ 1.26, d, J 6.6, 6- or 7-Me; 1.3, d, J 6.6, 7- or 6-Me; 2.34, s, 2-Me; 3.81, m, J 6.6 and 3.3, H6 and H7 (see Figure 4, p 105); UV: ε_{263} 8280 (4 mM HCl).

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one [46]: 4,5-Diamino-2-thioxopyrimidin-6(1H)-one[127] (2.5 g) in water (50 ml) and acetic acid (15 ml) was condensed with diacetyl (2.5 ml) by boiling under reflux for 1 h. The reaction mixture was cooled and the solids were filtered off, washed with water, ethanol and ether and then dried *in vacuo* to give 2.94 g of solid. This crude material was

recrystallized from methanol (300 ml) (charcoal) to give the title pteridine (1.97 g); m.p. >270^{0}C (dec.) (lit.[134,135] m.p. 280-285^{0}C). From the mother liquor a further quantity (0.49 g) of pteridine was obtained. (Found: C, 45.9; H, 3.9; N, 26.4; S, 15.6%. $C_8H_8N_4OS$ requires C, 46.1; H, 3.9; N, 26.9; S, 15.4%); ^{1}H NMR (2 M DCl): δ 2.64, s, 6- or 7-Me; 2.68, s, 7- or 6-Me; (1 M NaOD): δ 2.47, s, 6- and 7-Me.

cis-6,7-Dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one [27]: The preceding 6,7-dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one (0.5 g) in 1 M sodium carbonate (40 ml) was reduced by boiling under reflux with sodium borohydride (6 g total, added in several small portions) until the UV spectrum in 1.5 M HCl indicated complete reduction (15 h), and cooled. The solid (0.26 g) was collected, washed with water (centrifuge) and dried. The solid (0.15 g from 0.26 g of crude solid) was dissolved in methanol (15 ml), ethanol (15 ml) was added and the solution was filtered and cooled. The yellow solid was collected, washed with small volumes of methanol and dried to give colourless cis-6,7-dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(3H)-one (28 mg); m.p. >259^{0}C (dec.) (Found: C, 41.7; H, 5.6; N, 23.3; S, 13.2%. Calculated for $C_8H_{12}N_4OS$, $0.5CH_3OH$, $0.18NaH_2BO_3$, $0.1H_2O$: C, 41.6; H, 6.0; N, 22.8; S, 13.1%); ^{1}H NMR (0.01 M NaOD): δ 1.07, d, J 6.7, 6- or 7-Me; 1.08, d, J 6.7, 7- or 6-Me; 3.24,

octet, J 6.7 and 3.2, H6 or H7; 3.55, octet, J 6.7 and 3.2, H7 or H6 (see Figure 5, p 107); UV: ε_{282} 5342 (pH 0).

6,7-Dimethyl-2-thioxo-3,4,7,8-tetrahydropteridin-4(1H)-one [47]: To pre-reduced platinum oxide (50 mg) in 3 M HCl (5 ml), 6,7-dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one (50 mg) in 3 M HCl (5 ml) was added and hydrogenated for 5.5 h at 25^{0}C/720 mmHg. The catalyst was filtered off and the filtrate was evaporated to dryness to yield the title compound (68 mg). The ^{1}H NMR and UV spectra were characteristic for the 7,8-dihydro derivative. Also similar hydrogenations carried out in methanol (for 11 h) or trifluoroacetic acid (28 h), gave the same 7,8-dihydro derivative in both cases; ^{1}H NMR (5 M DCl): δ 1.55, d, J 7.1, 7-Me; 2.53, s, 6-Me; 4.94, q, J 7.1, H7.

2-Amino-6,7-dimethylpteridin-4(3H)-thione [48]: 2,4,5-Triaminopyrimidin-6(1H)-thione hydrochloride[158] which was supplied by Dr W.L.F. Armarego, (200 mg) was dissolved in water (5 ml), the pH was adjusted to 4 and diacetyl (0.2 ml) was added. The solution was boiled under reflux for 70 min. The solid (150 mg) that separated on cooling was collected and recrystallized from water (140 mg); m.p. >290^{0}C (dec.) (Found: C, 45.9; H, 4.4; N, 33.3; S, 14.5%. $C_7H_9N_5S$, $0.15H_2O$ requires C, 45.8; H, 4.5; N, 33.4; S, 15.3%); ^{1}H NMR (1 M DCl): δ 2.66, s, 6- or 7-Me; 2.68, s, 7- or 6-Me.

cis- and trans-2-Amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26a]:

i) Reduction with sodium borohydride: 2-Amino-6,7-dimethylpteridin-4(3H)-thione (30 mg) was reduced with sodium borohydride (200 mg) in 1 M sodium carbonate (3 ml) under nitrogen. The solution was boiled under reflux and after 45 min, 90 min and 12 h, further additions of 200, 100 and 150 mg of sodium borohydride respectively were made. The reaction was continued for 13 h. Ice-cold 7 M methanolic-HCl was added to the cooled mixture to acidify, and the solution was evaporated to dryness. This residue (2.33 g) was passed through a Dowex-50Wx4 (Sigma, 50 - 100 mesh, H^+ form, 2 ml) column, washed with 0.5 M to 1.5 M HCl and eluted with 2 M HCl. Evaporation of the last eluate gave a mixture of cis and trans isomers (60 mg); m.p. >211^{0}C (dec.), in the ratio of **1.6 : 1.0** by ^{1}H NMR (cf. Figure 6, p 109) (Found: C, 26.3; H, 4.7; Cl, 23.4; S, 9.5%. Calculated for $C_8H_{13}N_5S$, 1.6HCl, 0.8NaCl, 0.8H_3BO_3: C, 26.3; H, 4.7; Cl, 23.3; S, 8.8%); ^{1}H NMR (2 M DCl at 270 MHz assigned by proton decoupling) cis isomer: δ 1.33, d, J 6.8, 6- or 7-Me; 1.41, d, J 6.8, 7- or 6-Me; 3.98, m, J 6.8 and 3.3, H6 or H7; 4.05, m, J 6.8 and 3.3, H7 or H6 and trans isomer: δ 1.42, d, J 6.6, 6- or 7-Me; 1.53, d, J 6.6, 7- or 6-Me; 3.56, m, J 6.6 and 8.3, H6 or H7; 3.74, m, J 6.6 and 8.3, H7 or H6).

ii) Catalytic reduction with platinum oxide in trifluoroacetic acid: 2-Amino-6,7-dimethylpteridin-4(3H)-

thione (15 mg) in trifluoroacetic acid (2 ml) was added to pre-reduced platinum oxide (20 mg in 2 ml of trilfluoroacetic acid) and hydrogenated for 11 h at 25^{0}C/720 mmHg. The catalyst was filtered off through Celite and washed with methanol. The filtrate was evaporated to dryness *in vacuo*. The residue (23 mg, yellow oil) was purified from methanol and 7 M methanolic-HCl by addition of ether, and gave the reduced pteridine with a *cis* to *trans* ratio of **2.3 : 1.0** by ^{1}H NMR.

cis-2-Amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione [26b]: To pre-reduced platinum oxide (5 mg) in methanol (1 ml), 2-amino-6,7-dimethylpteridin-4(3H)-thione (5 mg) in methanol (1.5 ml) was added and the mixture was hydrogenated for 24 h as above. The catalyst was filtered off and the filtrate was poured into 7 M methanolic-HCl and evaporated to dryness *in vacuo*. This solid contained only *cis*-tetrahydropteridine isomer [26b] by ^{1}H NMR (see Figure 7, p 109).

6,7-Dimethyl-2-methylthiopteridin-4(3H)-one [50]: 6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1H)-one (5 g, 24.2 mmol) in 0.5 M NaOH (50 ml) was boiled under reflux with methyl iodide (4.42 ml, 33.9 mmol) for 15 min. The crystals [2.43 g; m.p. 280-284^{0}C (dec.)] were collected, washed with water and dried *in vacuo*. The mother liquor

was concentrated to a small volume and cooled to give more methylthiopteridine (1.0 g); m.p. >261^{0}C (dec.). The first crop (150 mg) was recrystallized from propan-1-ol (10 ml) to give pure 6,7-dimethyl-2-methylthiopteridin-4(3H)-one (106 mg); m.p. >255^{0}C (dec.) (lit.[134] m.p. 280-281^{0}C which was previously synthesized from 5,6-diamino-2-methylthio-pyrimidin-4(3H)-one, lit.[136] m.p. 283^{0}C) (Found: C, 49.0; H, 4.6; N, 25.4; S, 14.1%. $C_9H_{10}N_4OS$ requires C, 48.6; H, 4.5; N, 25.2; S, 14.4%); ^{1}H NMR (0.1 M NaOD): δ 2.54, s, CH_3S; 2.60, s(broad), 6- and 7-Me; (2 M DCl): δ 2.70, s, 6- and 7-Me; 2.83, s, CH_3S.

cis-6,7-Dimethyl-2-methylthio-5,6,7,8-tetrahydro-pteridin-4(3H)-one [28]:

i) Hydrogenation with platinum oxide in methanol: 6,7-Dimethyl-2-methylthiopteridin-4(3H)-one (50 mg, 225 μmol) in methanol (5 ml) was added to pre-reduced platinum oxide (45 mg) in methanol (5 ml), and hydrogenated (25^{0}C/720 mmHg) for 7.5 h. The catalyst was filtered off and the filtrate was evaporated to dryness and dried *in vacuo* to give cis-6,7-dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one (67 mg, 91.1%); m.p. >115^{0}C (dec.) (Found: N, 17.0; S, 9.5%. Calculated for $C_9H_{14}N_4OS$, $5.6H_2O$: N, 17.1; S, 9.8%); ^{1}H NMR ($CDCl_3$): δ 1.18, d, J 6.6, 6- or 7-Me; 1.22, d, J 6.6, 7- or 6-Me; 2.49, s, CH_3S; 3.49, octet, J 6.6 and 3.0, H6 or H7; 3.74, octet, J 6.6 and 3.3, H7 or H6 (see Figure 8, p 111); UV: ε_{274} 6710 (1.5 M HCl).

ii) Reduction with sodium borohydride: 6,7-Dimethyl-2-methylthiopteridin-4(3H)-one (20 mg, 90 µmol) and sodium borohydride (60 mg) were refluxed in iso-propanol (2 ml) for 2.25 h (after 70 min, another 20 mg of sodium borohydride was added). The reaction mixture was extracted with iso-propanol and the inorganic salts were removed by centrifugation. To the supernatant, 7 M methanolic-HCl was added, the solution was evaporated to dryness and then methanol and methanolic-HCl was added followed by ether. The first solid that separated was removed by centrifugation (inorganic salts) and the supernatant was evaporated to dryness and dried *in vacuo* to give cis-6,7-dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one (30 mg) (Found: C, 38.5; H, 5.6; Cl, 15.0; N, 18.7; S, 10.0%. Calculated for $C_9H_{14}N_4OS$, $0.1NaH_2BO_3$, 1.2HCl: C, 38.9; H, 5.6; Cl, 15.1; N, 20.2; S, 11.5%). The UV spectrum of this compound was identical with that from the above derivative. In the presence of acid this compound released methylthiol (odour), so presumably some decomposition occurred in acidic solution.

cis-6,7-Dimethyl-2-methylthio-[6,7-D_2]-5,6,7,8-tetrahydropteridin-4(3H)-one [29]: To pre-reduced platinum oxide (30 mg) in tetradeuteriated methanol (2.5 ml) and 6,7-dimethyl-2-methylthiopteridin-4(3H)-one [30 mg, which was dissolved in tetradeuteriated methanol (5 ml),

evaporated to dryness and then re-dissolved in tetra-deuteriated methanol (2.5 ml)] was added. Hydrogenation was carried out with D_2 gas for 23 h at 25^{0}C/720 mmHg. The catalyst was removed by filtration through Celite, evaporated to dryness and dried *in vacuo* to give the title compound (18 mg). Greater than 90% deuterium incorporation was demonstrated by ^{1}H NMR for this reduction.

6,7-Dimethyl-2-methylthio-7,8-dihydropteridin-4(3H)-one [51]: Pre-reduced platinum oxide (50 mg) in trifluoroacetic acid (5 ml), and pteridinone (50 mg) in trifluoroacetic acid (5 ml) were hydrogenated for 1.25 h as above. The reaction was followed by UV and ^{1}H NMR spectroscopy. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. Methanol and methanolic-HCl were added to it and the solid (12 mg) that was collected was examined by ^{1}H NMR and was starting material. The mother liquors were evaporated to dryness and the colour of the residual oil (45 mg) changed to dark green and had a slight odour of methylthiol on further drying. The ^{1}H NMR spectrum [(5 M DCl): δ 1.57, d, *J* 7.1, 7-Me; 2.58, s, C*H*$_3$S; 2.95, s, 6-Me; 4.99, q, *J* 7.1, H7] showed that it was mostly 6,7-dimethyl-2-methylthio-7,8-dihydropteridin-4(3*H*)-one.

cis-6,7-Dimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3H)-one [25] hydrochloride: This compound was supplied by Dr P. Waring and had ε_{270} 13,800 $M^{-1}.cm^{-1}$ (4 mM HCl) (Found: C, 35.4; H, 6.35; Cl, 22.8; N, 22.6%. $C_9H_{15}N_5O$, 2HCl, $1.4H_2O$ requires C, 35.3; H, 6.5; Cl, 22.8; N, 22.85%).

6,6,8-Trimethyl-5,6,7,8-tetrahydropterin [31] [126] was supplied by Dr D. Randles.

6,8-Dimethyl-5,6,7,8-tetrahydropterin [30] and **6-methyl-5,6,7,8-tetrahydrolumazine** [32] hydrochlorides were supplied by Dr W.L.F. Armarego.

3-6-3 The rate of rearrangement of quinonoid pteridines

The stock solution of each tetrahydropteridine was made in 4 mM HCl (pH ~2) to give a final concentration of ca 120 ~ 280 µM. All concentrations were determined from their absorbances and known ε values. The reaction mixture contained 1 M Tris/HCl (100 µl, pH 7.4) [or 1 M Bicine (100 µl, pH 7.1) or 3 M potassium phosphate (100 µl, pH 7.0)], substrate (100 µl), hydrogen peroxide (100 µl, 11 µmoles) and water (600 µl), and the reaction was initiated by addition of peroxidase (100 µl, 20 µg of Boehringer grade II enzyme or 40 µg of Sigma type I enzyme). The concentration of hydrogen peroxide was determined from its ε_{237} value of 67 $M^{-1}.cm^{-1}$ in water. The assays were

carried out with a single beam spectrometer and using the respective analytical wavelengths (cf. Table 1, p 115) at 25^{0}C. The values of k and $t_{1/2}$ were computed using a programme supplied by Dr D. Randles. The rates for the deuteriated analogues were measured in the same way and the deuterium isotope effects were calculated (Table 2, p 117).

The changes of UV spectra were measured on a Cary 219 spectrometer (automatic scanning) with the same (above) reaction cocktail (see Figures 9 and 10, p 113).

Potassium ferricyanide solution (100 µl, 200 µM) and water (700 µl) was used instead of the peroxidase and hydrogen peroxide solution when potassium ferricyanide was used as oxidant.

3-6-4 Non-enzymic oxidation of NADH

The solutions for the measurements of the rate of non-enzymic oxidation of NADH by quinonoid pteridinones generated in situ consisted of 1 M Tris/HCl buffer (100 µl, pH 7.4) (or 3 M potassium phosphate buffer, pH 7.1, 100 µl), peroxidase (100 µl, 20 µg of Boehringer grade II or 40 µg of Sigme type I enzyme), hydrogen peroxide (100 µl, 11 µmoles), substrate (in 100 µl of 4 mM HCl), and water (500 µl), and the reactions were initiated by addition of NADH (in 100 µl of 50 mM Tris/HCl buffer, pH 7.3). The rates were measured with a single beam spectrometer at 25^{0}C and 340 nm (Table 3, p 122).

When potassium ferricyanide was used to generate

the quinonoid species, the reaction was carried out in the following solution; 1 M Tris/HCl buffer (100 µl, pH 7.4), 2 mM potassium ferricyanide (100 µl, 200 µM), pteridine solution (50 or 100 µl in 4 mM HCl, <70 µM) and water (to make 1 ml in final volume). The reaction was initiated by addition of NADH solution (30 µl in 50 mM Tris/HCl buffer, pH 7.3). The difference in ε values between potassium ferricyanide and potassium ferrocyanide at 340 nm was small ($\Delta\varepsilon_{340}$ 210 $M^{-1}.cm^{-1}$) compared with the ε_{340} value of NADH (6,200 M^{-1},cm^{-1}). Therefore the effects of the absorbance changes due to the iron complex were very small compared with the changes for NADH and were neglected. Also in the kinetic assay, these effects were completely neglected because they were compensated in the two cuvettes (see below).

Moreover, the rates of oxidation of NADH with potassium ferricyanide were very small when the concentration of potassium ferricyanide was varied, i.e. this effect was neglected from the rate of non-enzymic oxidation of NADH.

3-6-5 The effect of borate on DHPR activity

The assay was carried out using a double beam UV spectrometer at 25°C with $\lambda_{analyt.}$ 340 nm. The assay solution contained 1 M Tris/HCl (100 µl, pH 7.4), peroxidase (Sigma, 100 µl, 40 µg), hydrogen peroxide (100 µl, 11 µmol), borate solution (100 µl), 6-MeTHP (5 µl, 55.0

μM), NADH (30 μl, 100 μM) and water (565 μl). The reaction was initiated by injecting the enzyme (1 μl, 0.04 μg) into one cuvette. Each borate stock solution was made in water to give final concentrations of 50, 100 μM and 1 mM of boric acid. The blank assay was performed with water instead of borate solution. All of above borate concentrations had no effect on the activity of DHPR.

The tetrahydropteridinones which contained borate were **5,6,7,8-tetrahydropteridin-4(3H)-one** [18] ($C_6H_8N_4O$, $0.1NaH_2BO_3$, H_2O), **[6,6,7,7-D_4]-5,6,7,8-tetrahydropteridin-4(3H)-one** [19] ($C_6H_4D_4N_4O$, $0.01NaH_2BO_3$, H_2O), **6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one** [20] ($C_7H_{16}N_4O$, $0.76NaH_2BO_3$, $0.1H_2O$), **6-methyl-[6,7-D_2]-5,6,7,8-tetrahydropteridin-4(3H)-one** [21] ($C_7H_8D_2N_4O$, $0.1NaH_2BO_3$, $0.9H_2O$) and **cis-6,7-dimethyl-2-thioxo-3,4,5,-6,7,8-hexahydropteridin-4(3H)-one** [27] ($C_8H_{12}N_4OS$, $0.5CH_3OH$, $0.18NaH_2BO_3$, $0.1H_2O$).

3-6-6 Enzyme assay and kinetic parameters

The assays were performed essentially as described by Nielsen et al.[9] in the first place (cf. Chapter 2, Section 2-9-1, p 73). The assay was initiated by injection of DHPR. With this method, the reproducibility was very poor, because of the instability of the quinonoid species of compounds [18], [20], [22] and [33]. To avoid that, the pteridine solutions and NADH solutions were each simultaneously added to both cuvettes by using two platinum

buckets which were then moved up and down (10 times) into the assay solutions in the sample and blank cuvettes. When the reaction was initiated by addition of pteridine solution or NADH solution, good reproducibility was obtained. To obtain the optimum enzyme concentration for each substrate, the assays were performed with different enzyme concentrations and keeping the concentrations of NADH and substrate constant (Figure 13, p 124). For the kinetic assays, the enzyme concentrations were taken from the linear portions of the plots of enzyme concentrations versus initial velocities (Figure 13).

Finally, the solutions for the kinetic runs for DHPR activity contained the following: 1 M Tris/HCl buffer (pH 7.4) (100 µl) [or 3 M potassium phosphate buffer (pH 7.1) (100 µl)], peroxidase (100 µl, 20 µg of Boehringer grade II or 40 µg of Sigma type I enzyme), hydrogen peroxide (100 µl, 11 µmol), DHPR (see Table 4, p 126, about concentration, added to one cuvette), NADH (30 µl, ca 100 µM), substrate (30 µl, concentration 0.5 ~ 3 Km) and water to make 1 ml. The first three components were made as a stock solution (kept at 0 ~ 2^0C during runs).

After mixing all ingredients except the substrate, the recorder was activated to ensure that the absorbance remained unaltered for approximately 30 sec. The reaction was initiated by simultaneous addition of pteridine solution to both cuvettes, and the initial rates were obtained from the rate of change of absorbance at 340 nm for NADH oxidation. Rates from duplicate runs from at

least five concentrations of substrates were used to derive the kinetic parmeters. All of kinetic parmeters were calculated using a computer programme that was kindly supplied by Dr A. Cornish-Bowden,[159] and values derived from double reciprocal plots of concentrations versus initial velocities agreed well with computed values. The pH values at the end of each run were measured with a glass electrode and were 0.1 pH unit lower than the values of the stock buffers originally used.

When the quinonoid species were generated by using potassium ferricyanide as an oxidant, the stock solution contained 1 M Tris/HCl buffer (100 µl, pH 7.4), 2 mM potassium ferricyanide (3 times higher concentration than the highest pteridine solution) and water (to make 1 ml in final volume). The pteridine solution (100 µl in 4 mM HCl, 0.1 ~ 1.5 K_m) and NADH solution (30 µl in 50 mM Tris/HCl buffer, pH 7.3) were each added simultaneously with platinum buckets which were then moved up and down (10 times) into the assay solution to ensure complete mixing. The reaction was initiated by addition of pteridine or NADH solution, and at the same time one of the buckets also contained DHPR (>1 µl). Soon after mixing the recorder was initiated. The loss of NADH concentration was small during the initial rate measurement but the concentration of NADH was still or near saturating levels (Table 3, p 122).

The app.K_m and app.V_{max} values for NADH were similarly measured using the following solution: 1 M Tris/HCl buffer (pH 7.4) (100 µl), peroxidase (Sigma type

I, 10 µl), hydrogen peroxide (100 µl, 11 nmol), DHPR (1 µl, 0.04 µg) and water (740 µl). The blank cuvette did not contain DHPR. NADH (30 µl) was added simultaneously (0.5 ~ 5 Km) to both cuvettes (sample and blank) and the reaction was initiated by the simultaneous addition of 6,7-dimethyl-5,6,7,8-tetrahydropterin stock solution (10 µl in 4 mM HCl, 50 µM) (see Table 6, p 131).

3-6-7 pH rate profile study

The 6,7-dimethyl-[6,7-D_2]-5,6,7,8-tetrahydropterin was used as substrate and quinonoid species were generated by peroxidase and hydrogen peroxide oxidation. Although the stabilities of quinonoid species were different at various pH values (Figure 14, p 136), the quinonoid species formed at neutral pH were stable in an ice-bath for at least 30 min. For example, pterin stock solution (in 4 mM HCl, 20 µl), 0.5 M Tris/HCl buffer (pH 7.2) (50 µl), peroxidase (Sigma type II, 100 µl), hydrogen peroxide (100 µl, 11 nmol) and water (730 µl) were mixed at room temperature. After the formation of quinonoid species this solution was placed in an ice-bath. Aliquots (50 µl) were taken and mixed with buffer cocktail (0.5 M Tris/HCl buffer, 100 µl; mixed buffer,[145] 100 µl; water 720 µl), NADH (30 µl, 141 µM) and the reaction was initiated by injection of DHPR (5 µl, 0.2 µg) (Figure 15, p 137). [The purpose of the **mixed buffers** was to produce sufficient buffering action with varying pH values and consisted of 1

M MES and 0.5 M ethanolamine and adjusted to the required pH with HCl or NaOH solution. Tris/HCl buffer was added separately. In the final assay cocktail, the concentration of each ingredient was 50 mM Tris, 100 mM MES and 50 mM ethanlamine and the ionic strength was kept at ca 0.2 by adding NaCl to each different pH buffer stock solution. The pH of the stock solutions were pH 5.81, 6.24, 6.88, 7.87, 8.56, 9.21 and 9.88, and in the assay mixture the final pH was always 0.1 pH unit lower than the above.]

The stability of quinonoid species at different pH values was measured by addition of quinonoid solution formed by peroxidase and hydrogen peroxide oxidation (900 µl) to each buffer mixture (100 µl) at 25^0C using a single beam spectrometer (Figure 14, p 136).

The rates of non-enzymic oxidation of NADH at different pH values were measured by initiating the reaction by adding NADH (30 µl, 152 µM) to the quinonoid solution (100 µl, 61 µM as tetrahydro derivative), 0.5 M Tris/HCl buffer (100 µl), mixed buffer (100 µl) and water (670 µl) at 25^0C using a single beam spectrometer and observing the absorbance change at 340 nm (Figure 15, p 137).

It was not possible to obtain reliable kinetic parameters (app.K_m and app.V_{max}) at the various pH values, because the reproducibility of initial rates was very poor.

3-6-8 **Attempted identification of the oxidation products of 4,5-diaminopyrimidin-6(1H)-one [33] and pteridinones [18], [20], [22]**

A: by TLC and NMR

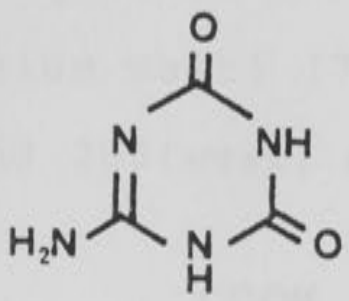
i) Peroxidase (100 µl), hydrogen peroxide (100 µl, 3.3 %), 1 M Tris/HCl buffer (100 µl), pteridinone solution (see below) and water (to make a total volume of 1 ml) were mixed at 20°C and aliquots were taken at time intervals and placed on TLC plates (p 152). In the case of 4,5-diaminopyrimidin-6(1H)-one [33] (100 µl, 350 µM), at least five spots including spots from the blank (peroxidase, hydrogen peroxide and Tris; two main spots) were observed. The number of spots had not changed between 1.5 min and 7 h incubation periods. None of them had the same characteristics as 2-carboxy-1,3,5-triazin-4(3H)-one [66] (p 141) or 1,3,5-triazin-2,4(1H,3H)-dione [67] (p 141). In the case of tetrahydropteridin-4(3H)-one [18] (60 µl, 1.97 mM) eight spots were obtained including the spots from the blank. One blue fluorescent (250 nm lamp) spot (Rf ~0.9) was extracted and gave a solution with only "tail" absorption in the UV spectrum. The fluorescence of this spot increased with time. This may be due to dimeric pteridine species. In the case of 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one [20] (30 µl, 1.19 mM), seven spots were revealed. Also the same blue fluorescent spot appeared (Rf ~0.9). In the case of 6,7-dimethyl-5,6,7,8-

tetrahydropteridin-4(3H)-one [22] (100 µl, 585 µM), six spots appeared after 1 min and the relative intensities, but not the number of spots, altered with time. Attempts to identify the spots in those solutions were uniformly unsuccessful. Most of the spots had "tail" or no UV absorption. Only traces of the fully oxidized pteridines were identified. The oxidation is not clear and produces a variety of products from the early stages of the reaction.

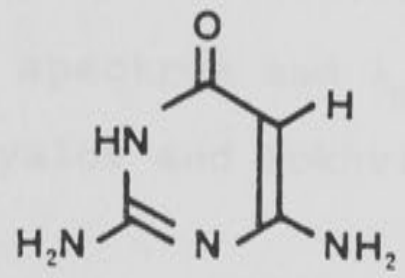
ii) 6-Methyl-5,6,7,8-tetrahydropteridin-4(3H)-one [20] (50 mg, 0.215 mmol) was mixed with 30% hydrogen peroxide (0.2 ml) and stirred at 20°C. After 10 h, the solids were removed (<1 mg) by filtration and the filtrate was freeze-dried (60 mg). The residue (10 mg) was dissolved in water and separated by TLC (cf. p 152). The six bands were extracted with 1 : 1, methanol - methylene chloride and examined by UV and ^{1}H NMR spectroscopy. Only 6-methylpteridin-4(3H)-one [37] (<10% yield) was fully identified.

iii) 4,5-Diaminopyrimidin-6(1H)-one [33] (100 mg) in 30% hydrogen peroxide (0.5 ml) was stirred for 15 h at 20°C. Some ammonia was released (odour). The solids (47 mg, m.p. >300°C) that separated were collected and showed similar Rf values (~0.5) on TLC as the one from 2-amino-1,3,5-triazin-4,6(1H,5H)-dione (Ammelide)[147] [77] which was obtained from the reaction of 2,4-diamino-pyrimidin-6(1H)-one [78] and hydrogen peroxide. The

reported λ_{max} value of Ammelide is 221 nm (in water).[148]

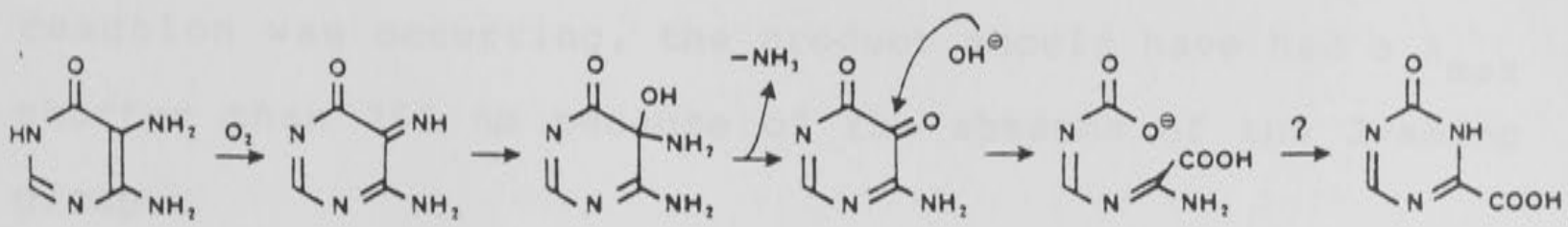


[77]



[78]

On the other hand, the solid obtained had λ_{max} at ca 220, 280 (weak) and 330 (very weak) nm (in water), and gave very strong yellow fluorescence when it was dissolved into 10 M NaOH solution. The UV spectrum indicates that perhaps the pyrimidine ring was cleaved and that another UV absorbing heterocyclic compound was formed (e.g. Scheme 10).

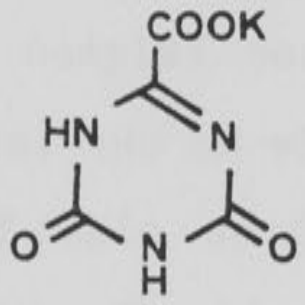


[33] [69] [66]

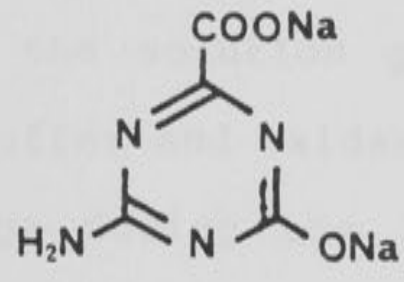
Scheme 10

iv) A mixture of 4,5-diaminopyrimidin-6(1H)-one [33] (100 mg), 30% hydrogen peroxide (0.5 ml) and 2 M NaOH (1.25 ml) was stirred for 3 h at 20°C. Ammonia was released (odour) and the yellow solids formed were collected (10 mg); m.p. >300°C. The filtrate was further reacted with 30% hydrogen peroxide (0.2 ml) for 2 h and more yellow solids were collected (50 mg); m.p. >300°C. On

TLC, none of spots had the same Rf as authentic 4,6-dihydroxy-1,3,5-triazine-2-carboxylic acid (oxonic acid, potassium salt) [79], and the UV spectrum had λ_{max} at ca 220 and 280(weak) nm (pH 7). Zav'yalov and Pokhvisneva[147]



[79]



[80]

reported that sodium 4-amino-6-hydroxy-1,3,5-triazine-2-carboxylate [80], which was obtained from the 2,4-diamino-pyrimidin-6(1H)-one [78], had λ_{max} at 250 nm (in water) and m.p. >300^{0}C (dec.). If in the present case the same reaction was occurring, the product should have had a λ_{max} shorter than 250 nm because of the absence of the 2-amino group.

v) 4,5-Diaminopyrimidin-6(1H)-one [33] (3 mg) was dissolved into D_2O (2 ml) and aliquots of this solution (0.6 ml) were added to 3.3% hydrogen peroxide in D_2O (0.15 ml) and peroxidase (1 mg in 2.5 ml of D_2O, 1 drop) in a NMR tube. After 16 min, the signal from the C2 proton disappeared which was indication of exchange with deuterium or degradation of the pyrimidine followed by deuterium exchange (Figure 16, p 140).

vi) 6,7-Dimethyl-5,6,7,8-tetrahydropteridin-

4(3H)-one [22] solution (30 µl, 147 µM) was mixed with 1 M Tris/HCl buffer (pH 7.6) (100 µl), 5 mM potassium ferricyanide (100 µl, 500 µM) and water (770 µl) at 20°C and aliquots were taken at various time intervals and were examined by TLC. Soon after mixing, the yellow colour of the iron complex became weaker and the solution gave six main spots, two of which were from buffer and oxidant. The number of main spots did not change during the 18 h of incubation. The blue fluorescent spot at the solvent front was similar to the one from the peroxidase and hydrogen peroxide oxidation above. Only traces of 6,7-dimethylpteridin-4(3H)-one [36] (p 99) were identified in the products after 18 h. As above the other bands gave unidentifiable products which had no maximum in the UV spectra suggested that they did not contain an unsaturated heterocyclic chromophore. Only traces of these pteridinones were following the same oxidation pathway as the pterins to give fully oxidized pteridinones.

vii) cis-6,7-Dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one [27] (p 96) (1.5 mg, 1.4 mM) was mixed with 1 M Tris/HCl buffer (pH 7.4) (0.5 ml) and water (3.25 ml) and then potassium ferricyanide (1.25 ml, 5 mM) was added. The reaction was followed by TLC. Soon after addition of oxidant the spot from tetrahydro derivative disappeared, and after 40 min another spot which had the same Rf as fully oxidized pteridinone appeared. This spot became stronger with time. After 1 h, this spot

was the main spot on TLC which had two other weak unidentifiable spots.

B: The reaction with horseradish peroxidase and hydrogen peroxide

The kinetic runs for obtaining the K_m and V_{max} values of peroxidase in the presence of hydrogen peroxide at 25^0C were performed with 1 M Tris/HCl buffer (pH 7.4) (100 µl) [or 3 M potassium phosphate buffer (pH 7.1) (100 µl)], substrate (100 µl, 0.5 ~ 2 Km concentration), hydrogen peroxide (100 µl, 11 or 22 nmol; the concentration of stock solution was determined from the ε_{237} value of 67 $M^{-1}.cm^{-1}$ in water) and water to make 990 µl (or 1 ml in the blank cuvette). The reaction was initiated by injection of peroxidase (10 µl, Boehringer, 2 µg) to the reaction cuvette and the initial rates were obtained by measuring the rate of decrease in absorbance at an analytical wavelength set for each substrate (see Table 7, p 146). When measuring the effects of each component, the concentration of hydrogen peroxide or peroxidase was altered by changing the amount of hydrogen peroxide or volume of enzyme solution repectively (see Figure 17, p 145). The non-enzymic rates, in 0.1 M Tris/HCl buffer (pH 7.4) were negligible for compounds [18], [20], [22] and [33] in the absence of hydrogen peroxide and 8.9, 7.0, 7.6 and 5.9% substrates [i.e. tetrahydropteridin-4(3H)-ones [18], [20], [22] and [33]] were oxidized respectively in

the first 10 min in the presence of 11 mM hydrogen peroxide alone. The amounts oxidized in the first 10 min were slightly less than above, when hydrogen peroxide was 11 μM. The kinetic parameters (K_m and V_{max}) were calculated using a computer programme[159] (see Table 7, p 146).

CHAPTER 4

INHIBITION OF DIHYDROPTERIDINE REDUCTASE FROM HUMAN BRAIN

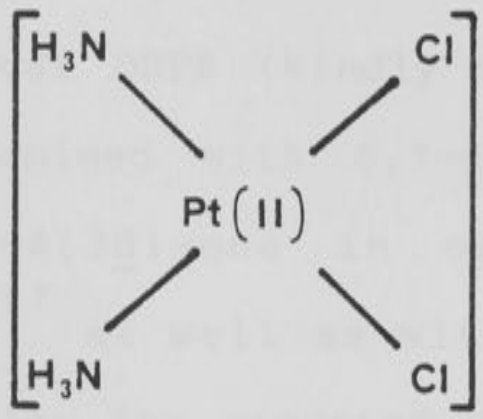
4-1 Introduction

Inhibitors of human DHPR have been reported by several authors. Shen et al.[90,97] have described the inhibition of human liver DHPR by a variety of catecholamines and related compounds including dopamine. Armarego and Waring[15,160] have shown that the true catecholamine inhibitors are in fact the oxidation products of the catecholamines, the quinone dopachromes, generated by horseradish peroxidase and hydrogen peroxide in the assay procedure used by Shen et al.[90,97] However, Shen et al.[96,101~104] have also described several other phenolic compounds which are true inhibitors. These are not readily oxidized under the assay conditions and are known to be inhibitors for other enzymes involved in the metabolism of catecholamines (e.g. monoamine oxidase). Other authors[90] also had shown that aminopterin, which is a strong tight binding inhibitor of dihydrofolate reductase is also an inhibitor of human liver DHPR, albeit a weaker inhibitor.

Firgaira et al.[10] reported briefly the inhibition of human liver DHPR by thiol specific reagents and metal ions, for example, $HgCl_2$ (0.1 μM; 60%), p-chloromercuri-benzoate (10 μM; 70%) and 5,5'-dithiobis(2-nitrobenzoic acid) (100 μM; 60%) which markedly inhibited the enzyme, whereas N-ethylmaleimide (1 mM; 76%) and iodoacetamide (10 mM; 70%) showed weaker effects. The use of these compounds at concentrations one order magnitude higher than those shown above produced total inhibition of activity.

However, $MgCl_2$ (10 mM) and $MnCl_2$ (10 mM) had no effect on enzyme activity, but $CoCl_2$ caused 30% inhibition at a concentration of 0.01 mM. On the other hand, Williams et al.[51] found that DHPR from a Pseudomonas species was inhibited by $MgCl_2$, $MnCl_2$ and $CdCl_2$ but not by $CoCl_2$.

Dhondt et al.[95], Purdy et al.[93] and Leeming et al.[94] described the inhibition of DHPR (from rat tissues) by metals (Pt, Pb and Al respectively) and stated that the metals may cause significant inhibitions also of the human enzyme which may be serious because these metals could accumulate in the body either accidentally or via drug therapy using these metals e.g. cis-platin (cis-diaminodichloroplatinum) [1].



[1]

The strongest known inhibitors of human DHPR have Ki values in the micromolar range and a search for more potent inhibitors is required for the preparation of active site directed irreversible inhibitors for determining amino acid residues at or close to the active site (affinity labelling reagents), and for inducing DHPR deficiency in experimental animals so as to mimic hyperphenylalaninemia due to this cause.

4-2 Inhibition by oxidized pteridines

Cheema et al.[17] reported that 6,7-dimethylpterin (app.Ki 300 μM) and 6,7-dimethylpteridin-4(3H)-one (app.Ki 50 μM) showed non-competitive inhibitior activities for sheep liver DHPR. In the present work, pteridin-4(3H)-one and 6,7-dimethylpteridin-4(3H)-one were examined for their inhibitory effect on human brain DHPR. These compounds were pre-incubated for some time (e.g. 30 min) with the enzyme in potassium phosphate buffer (pH 7.3) and then assayed with quinonoid 6-methyl-7,8-dihydro(6H)pterin and NADH. They exhibited no inhibitory effect on human brain DHPR (5.6 μM) at inhibitor concentrations up to 206 μM and 185 μM respectively at pH 7.3.

Sheep liver DHPR (kindly supplied from Dr W.L.F. Armarego) was examined with 6,7-dimethylpterin and 6,7-dimethylpteridin-4(3H)-one in order to compare with Cheema's report,[17] as well as with 6,7-dimethyl-2-thiolumazine. To follow the reported experimental conditions, 6,7-dimethyltetrahydropterin was used as a substrate precursor but potassium ferricyanide [$K_3Fe(CN)_6$] was used as oxidant to generate the quinonoid substrate. The iron complex was a more convenient oxidant than dichlorophenol-indophenol which was used in the reported work. 6,7-Dimethylpterin at 447 μM, 6,7-dimethylpteridin-4(3H)-one at 111 μM and 6,7-dimethyl-2-thiolumazine at 211 μM exerted 50%, 0% and 20% inhibition of sheep liver DHPR respectively after incubation for 3 min. These results for 6,7-

dimethylpterin were consistent with those reported, but the non-inhibitory effect observed with 6,7-dimethylpteridine-4(3H)-one was different. Cheema et al.[17] stated that the latter substance showed non-competitive inhibition with sheep liver DHPR. There are small differences between the present and the reported methods, e.g. the oxidants, the sources of the pteridines (i.e. syntheses) and the buffers used, but it is unlikely that these are the reasons for the large difference in the results obtained. No plausible explanation can be given at present for the discrepancy.

The inhibitions of human brain DHPR by the pterins, pteridinones and the thio-derivative studied were very weak or absent, and further studies along those lines were discontinued.

4-3 Inactivation by potassium ferrocyanide

When DHPR activity is assayed with a quinonoid dihydro(6H)pterin substrate which is generated by $K_3Fe(CN)_6$ oxidation of the tetrahydropterin, we are concerned that the DHPR activity may be affected by $K_3Fe(CN)_6$. This oxidant could conceivably inhibit the enzyme by reacting with amino acid residues via electrophilic attack or it may oxidize some amino acid residues.[16]

$K_3Fe(CN)_6$ was examined at concentrations of up to 30 mM, but showed no effect on the human brain DHPR activity when it was pre-incubated with DTT free enzyme. On the other hand, potassium ferrocyanide [$K_4Fe(CN)_6$] at 10

- 30 mM concentrations affected human brain DHPR activity. Up to ca 90% inhibition was observed after 2 h at the above concentration. The remaining 10% of enzyme activity may be due to protection by traces of bound NADH or deterioration of the enzyme-inhibitor complex by oxidation of the active inhibitor with time. Dialysis of these incubation solutions did not help to recover the original enzyme activities, i.e. the inactivations included an irreversible step. Dithiothreitol (2.6 mM) had a slight protective effect on this inactivation when the $K_4Fe(CN)_6$ concentration was 20 mM, and the protection by NADH was better at higher NADH concentrations (Figure 1).

If the inactivation process involved a fast pre-equilibrium between the enzyme and inhibitor prior to showing irreversible inhibition, i.e. $k_{-1} >> k_2$ then the

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI' \qquad (1)$$

reaction rate should be controlled by k_2 and exhibit first order kinetics. This was not the case here and a plot of the log values of the remaining activities versus incubation times gave curved lines, i.e. this inactivation by $K_4Fe(CN)_6$ did not follow the simple first order kinetics law, and is clearly more complicated than shown in Equation (1) (cf. time dependent inactivation by K_2PtCl_4, p 207).

In a fluorescence study, the inhibition of human brain DHPR by $K_4Fe(CN)_6$ showed time dependent quenching of

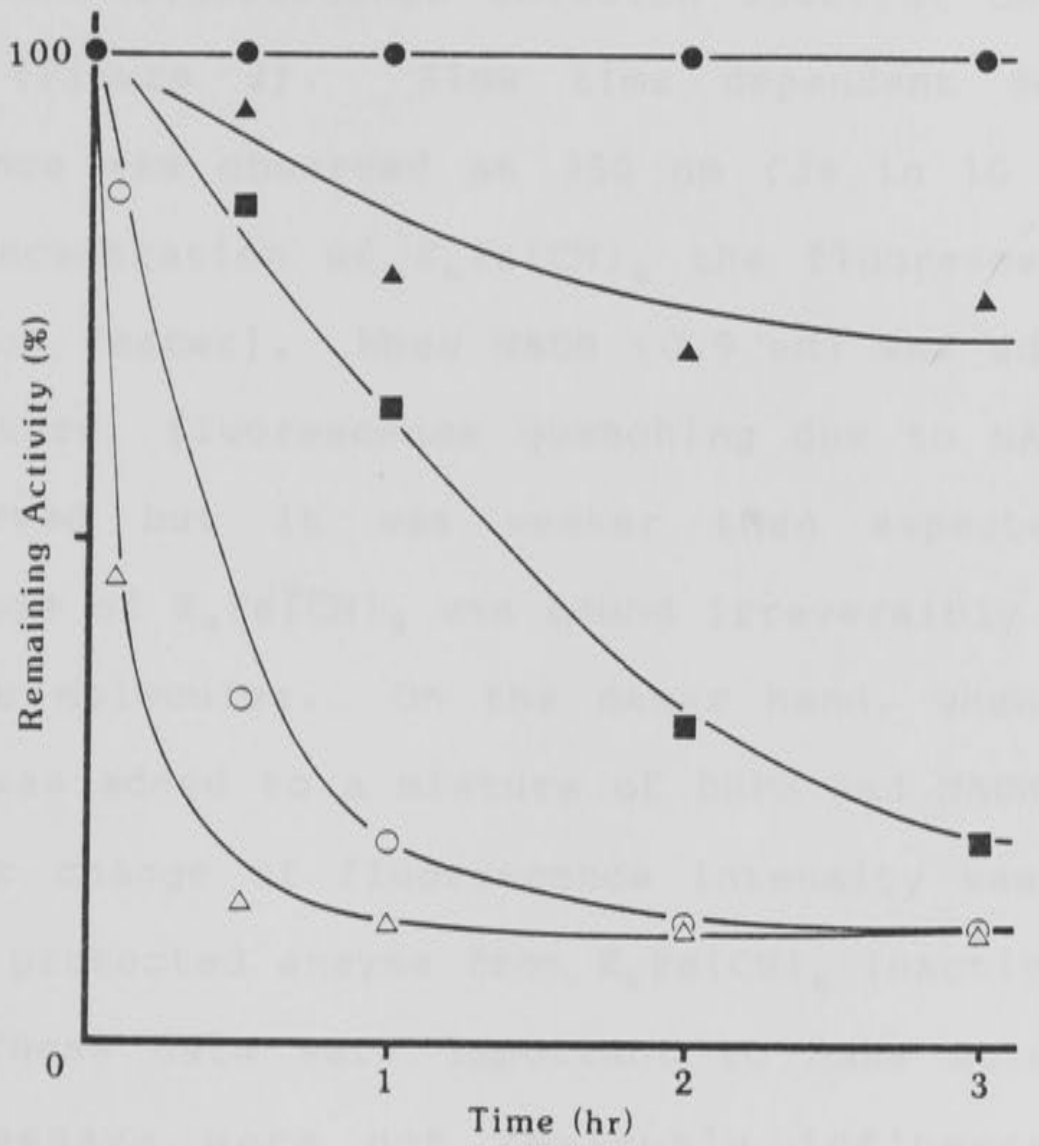


Figure 1. Time dependent inactivation of human brain DHPR by $K_4Fe(CN)_6$ (20°C, pH 7.2). All incubation mixtures contained 3.60 µM of DHPR. -●-●- in 0.03 M potassium phosphate buffer (pH 7.2); -▲-▲- 100 µM NADH and 20 mM $K_4Fe(CN)_6$ in the same buffer; -■-■- 5.5 µM NADH and 20 mM $K_4Fe(CN)_6$ in the same buffer; -○-○- 10 mM $K_4Fe(CN)_6$ in the same buffer; -△-△- 20 mM $K_4Fe(CN)_6$ in the same buffer.

enzyme fluorescence. DHPR (DTT free) was mixed with $K_4Fe(CN)_6$ solution in 0.1 M potassium phosphate buffer (pH 7.2) and the fluorescence emission spectral changes were observed (Figure 2). Slow time dependent decrease in fluorescence was observed at 350 nm (3% in 10 min) [with higher concentration of $K_4Fe(CN)_6$ the fluorescence change became much faster]. When NADH (0.9 μM) was added to the above mixture, fluorescence quenching due to NADH binding was observed but it was weaker than expected because already some of $K_4Fe(CN)_6$ was bound irreversibly to some of the enzyme molecules. On the other hand, when $K_4Fe(CN)_6$ (100 μM) was added to a mixture of DHPR and NADH (100 μM), no further change of fluorescence intensity was observed, i.e. NADH protected enzyme from $K_4Fe(CN)_6$ inactivation.

These data were important to make sure that the routine assays were not seriously influenced by the presence of these iron complexes. It should be pointed out that the concentration of $K_4Fe(CN)_6$ which is derived from $K_3Fe(CN)_6$ at the beginning of the usual assay is very low and its effects on the initial rates would be minimal.

The reason for the differences in reactivity between $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ on DHPR activity is possibly because Fe^{2+} can react with the protein at the His, Asp, Glu, Cys, Lys, or Arg residues. According to Pearson,[162] Fe^{2+} has higher affinity for the RSH group than Fe^{3+}. If the reactivity of Fe^{2+} towards RSH or RS^-, etc. is higher than with CN^-, Fe^{2+} may react irreversibly with these residues on the protein.

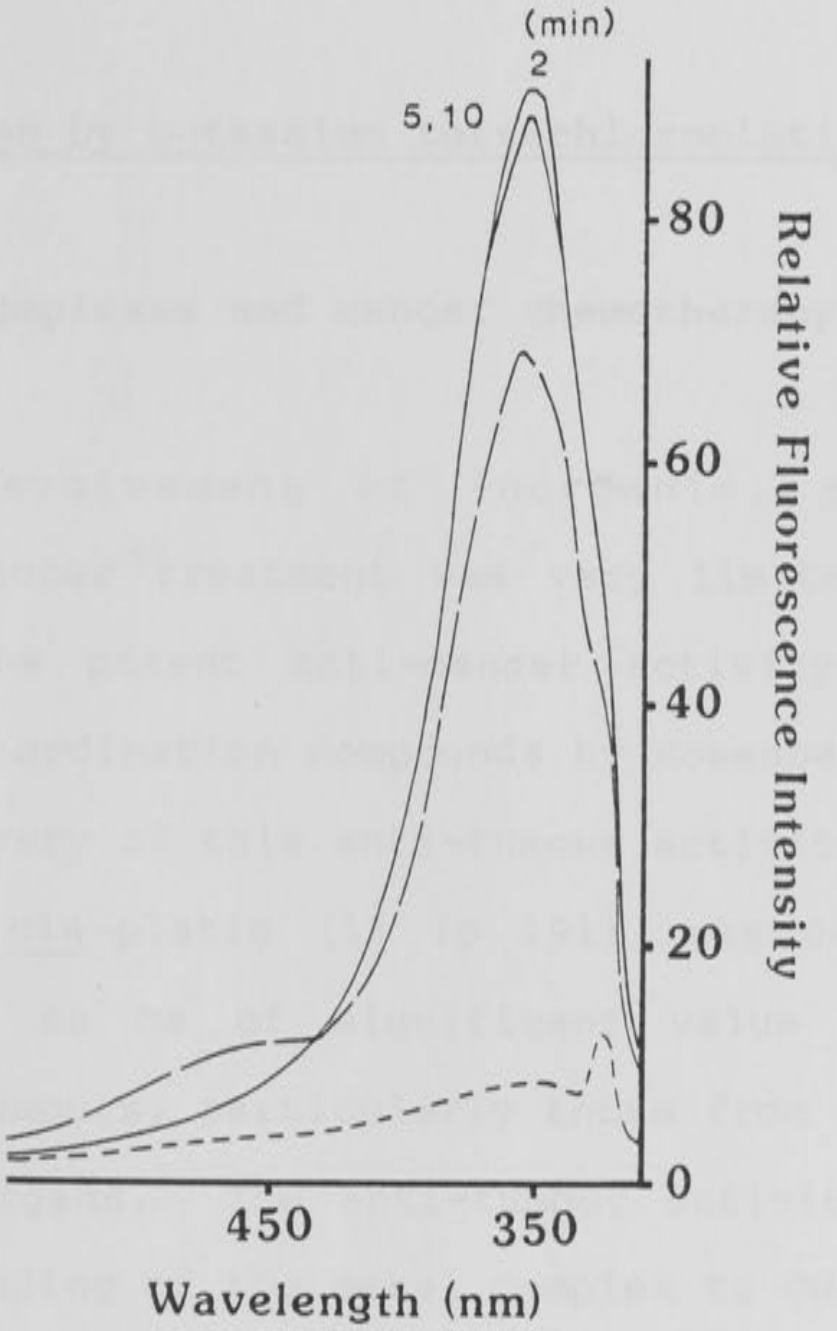


Figure 2. Fluorescence emission spectra of DHPR with $K_4Fe(CN)_6$ at 20°C, pH 7.2. ——— $K_4Fe(CN)_6$ (100 µM) in 0.1 M potassium phosphate buffer (pH 7.2). The emission spectra were scanned at time intervals (excitation wavelength was 280 nm); — — After 10 min, NADH (4 µM) was added (no change after 2 min); - - - $K_4Fe(CN)_6$ (100 µM) in 0.1 M potassium phosphate buffer (pH 7.2).

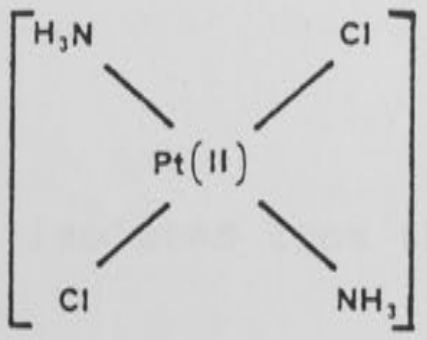
No formation of enzyme dimer occurred in the above inactivated enzyme as shown by SDS-PAGE, implying that this inhibitor does not cause intermolecular crosslinking.

4-4 Inactivation by potassium tetrachloroplatinate

4-4-1 Pt(II) complexes and cancer chemotherapy

The involvement of inorganic, metal-based compounds in cancer treatment was very limited until the discovery of the potent anti-cancer activity in certain platinum(II) coordination compounds by Rosenberg et al.[163] After the discovery of this anti-tumour activity, the first platinum drug, cis-platin [1] (p 191), was developed and has now proved to be of significant value in treating several human tumours, particularly those from the head and genitourinary organs. The anti-tumour activity was shown to be due to binding of the metal complex to DNA.[164,165]

However, cis-platin was found to cause cytotoxic damage especially to the kidneys (Table 1). To minimize this cytotoxicity and to raise membrane permeability, several other Pt(II) complexes were developed, but cis-platin is still the most widely used drug. Moreover, in these complexes, only the cis and non-electrically charged



[2]

Table 1. Organ distribution of cis-platin in rat tissues.[166]

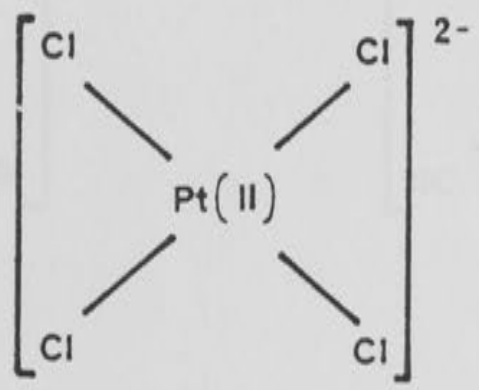
organs	% of injected dose/g of tissue
Blood*	0.378
Skin*	0.495
Thyroid	0.119
Liver*	0.755
Spleen	0.334
Pancreas	0.306
Stomach	0.110
Small intestine	0.411
Large intestine	0.351
Kidneys	**1.747**
Adrenals	0.614
Heart	0.166
Lung*	0.569
Brain*	0.035
Muscle	0.002
Tumour	0.226
Bone	0.584
Marrow	0.234
Bladder	0.520
Tendons	0.674

* DHPR had been isolated from these human tissues.[22]

(i.e. neutral) isomers (e.g. [1]) had anti-tumour activities; the trans isomers (e.g. [2]) were much less effective.

4-4-2 Pt(II) complexes and other enzymes

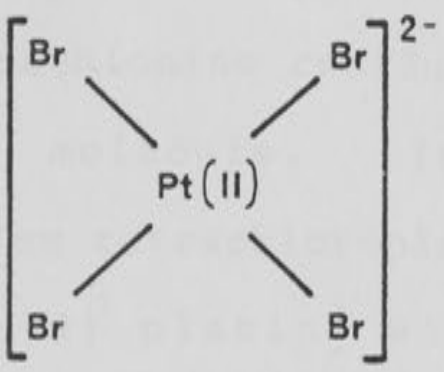
Platinum complexes have been widely used in the X-ray crystallographic studies of the three-dimensional structures of proteins with which they complexed.[167] Several amino acids were found to form complexes with Pt(II) complexes at reactive sulphydryl groups in proteins,[168] and the tetrachloroplatinate ion ($PtCl_4^{2-}$) [3] was found to preferentially bind to methionine residues which reacted with Pt(II) complexes by nucleophilic attack by the sulphur atom.[169] It is known that different platinum (Pt) complexes have different binding sites on proteins, although the sites have not yet been identified directly except in studies by X-rays.



[3]

In the interactions between Pt complexes and proteins, it has been stated that negatively charged complexes are more effective binders than the neutral or positively charged complexes.[170] This is in contrast to

the reactions with nucleic acids in which the neutral Pt complexes appear to react better (perhaps by intercalation). The ligands on the Pt(II) atom showed different effects on the rates of interaction between Pt(II) complexes and proteins. Friedman et al.[171] showed that tetrabromoplatinate ion ($PtBr_4^{2-}$) [4] inhibited malate dehydrogenase more strongly than $PtCl_4^{2-}$ [3], i.e. the reaction depended on the release of a ligand group from the Pt complexes in aqueous buffers. This phenomenon was explained as follows; the real inhibitors were the aquo species rather than tetrahalo compounds,[172] and bromide ligands on the platinum complex could be exchanged with water much faster than chloride ligands, and these Pt-aquo species will exchange their water ligands with some amino acid residues on proteins. Teggins et al.[171,173] reported that bromide ligands were more labile than chloride ligands



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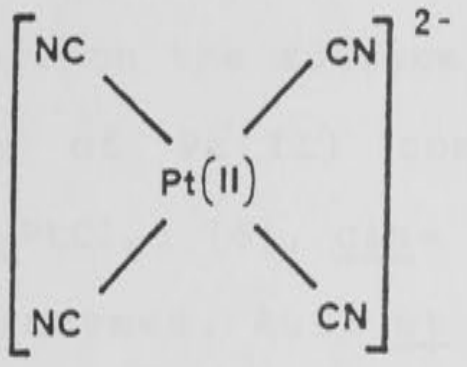


[5]

in substitution reaction of these Pt(II) complexes in their malate dehydrogenase inhibition studies. However, in general it is known that the affinity of Pt(II) metals for several ligands is in the order R_2S, RSH, RS^- > RNH_2, NH_3 > Br^- > Cl^- > H_2O[174] and this is also approximately the order

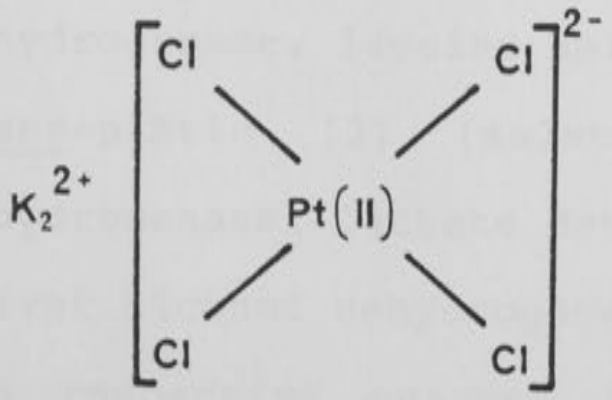
of decreasing <u>trans</u>-effects of these ligands. Moreover, complexes such as tetracyanoplatinate ion [$Pt(CN)_4^{2-}$] [5] with more stable ligands can bind proteins electrostatically (i.e. no ligand exchange), while complexes such as $PtCl_4^{2-}$ generally form covalent protein linkages.[174]

In aqueous solution, Pt(II) complexes (e.g. $PtCl_4^{2-}$) undergo ligand-exchange with water molecules and the equilibria in Scheme 1 are set up. Although X-ray

$$PtCl_4^{2-} \overset{H_2O}{\rightleftharpoons} PtCl_3(H_2O)^- \overset{H_2O}{\rightleftharpoons} PtCl_2(H_2O)_2 \overset{H_2O}{\rightleftharpoons} PtCl(H_2O)_3^+ \overset{H_2O}{\rightleftharpoons} Pt(H_2O)_4^{2+}$$

Scheme 1

analysis of Pt binding sites on the enzymes were carried out in the solid state, Dickerson <u>et al.</u>[175] showed that in these enzymes the preferential binding site of $PtCl_4^{2-}$ was at the methionine residues which are on the surface of the protein molecule. In a study of Pt(II) complexes [potassium tetrachloroplatinate (K_2PtCl_4) [6], <u>cis</u>- [1] and <u>trans</u>- [2] platin] with seven enzymes, Aull <u>et al.</u>[176] reported that the enzymes which had no cysteine residues



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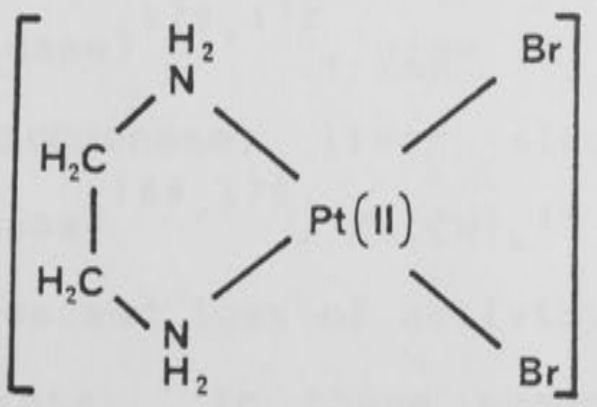
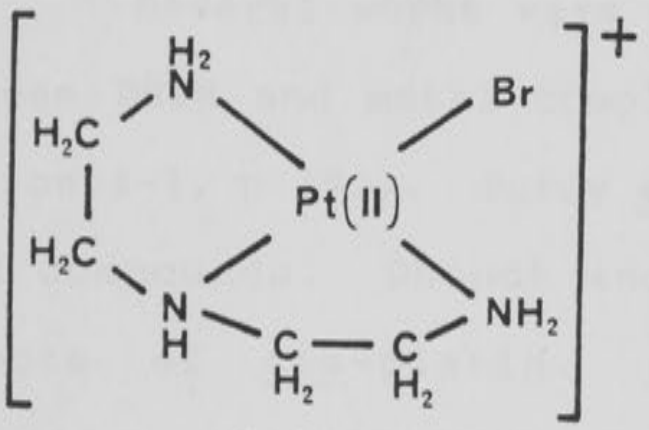


[7]

were not inhibited, whereas those possessing cysteine residues were inhibited. There was one exception, however, leucine aminopeptidase,[177] which was known to be a metal-requiring enzyme and possessed no essential thiol residues, was inhibited by $PtBr_4^{2-}$ [4] (100% inhibition at 5 mM, after 1 h), cis-dibromo[ethylenediamine]platinum ($Pt[en]Br_2$) [7] (80%; 5 mM; 50 h) and bromo[diethylenetriamine]platinum ($Pt[dien]Br^+$) [8] (20%; 5 mM; 50 h). Also mono-aquo tribromoplatinate [$PtBr_3(H_2O)^-$] [9], which was



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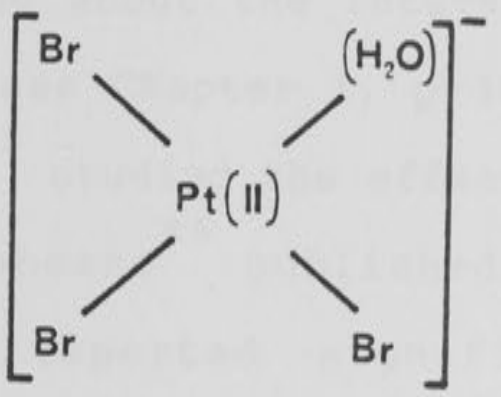


[9]

formed by aging the aqueous solution showed stronger inhibitory effect than $PtBr_4^{2-}$. In this case the interaction may have been with the ω-NH_2 group of e.g. a lysine residue, or the -SMe group of a methionine residue at or near the active site.

In summary the Pt(II) complexes of, for example, $PtCl_4^{2-}$ [3] (malate dehydrogenase)[170], $PtBr_4^{2-}$ [4] (malate dehydrogenase, leucine aminopeptidase)[170,172], cis- [1] and trans-platin [2] (malate dehydrogenase, liver alcohol dehydrogenase, lactate dehydrogenase)[168,170], $Pt(CN)_4^{2-}$ [5] (liver alcohol dehydrogenase)[178] caused loss of activity of the respective enzymes in brackets. In these examples inactivation followed first order kinetics and the

equilibrium constants k_{-1}/k_1 (Equation 1) were reported. In examples where the inhibition was irreversible, e.g. $Au(CN)_2^-$ (liver alcohol dehydrogenase)[178] and trans-platin (thymidylate synthase)[179], the inhibition did not follow simple first order kinetics.

4-4-3 Inactivation studies of human brain DHPR with different Pt(II) complexes

Several works were reported about the interaction between DHPR and metal complexes (see Chapter 1, p 30 and Section 4-1, p 190). Purdy et al.[93] studied the effects of lead compounds. Dhondt and Bellahsene[95] published some effects of cis-platin. They reported significant inhibition of DHPR (both worked on rat enzyme), although the experimental details of the latter authors were not clear. If human DHPR behaves in the same way, then cis-platin chemotherapy may cause DHPR deficiency in patients, although cis-platin does not enter brain easily.[95] DHPR from human brain and other parts of the body is genetically the same, and all human DHPR seem to have similar properties (cf. Chapter 2, p 37 or reference 22). The human brain enzyme was chosen for the present studies because it is more readily available to us and also because brain is relatively free from infectious diseases (cf. Table 1, p 199).

A detailed study of the inactivation of human brain DHPR by K_2PtCl_4 [6] is presented in this chapter together with a comparison of the effects of cis- and

trans-platin and $Pt[en]Cl_2$ complexes. Also the reactivity of the thiol groups in enzyme-Pt(II) complexes has been investigated, because this may provide information about the nature of the cysteine residues in the protein.

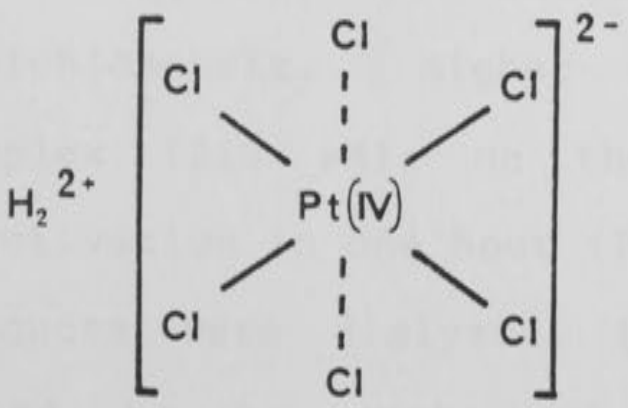
A comparative study of the effects of various concentrations of cis-platin, trans-platin, $Pt[en]Cl_2$ and K_2PtCl_4 on the inactivation of human brain DHPR is summarized in Table 2.

Table 2. The inhibition of DHPR by different Pt(II) complexes.

Time	Remaining activity (%)					
	K_2PtCl_4	trans-$Pt(NH_3)_2Cl_2$		cis-$Pt(NH_3)_2Cl_2$		$Pt[en]Cl_2$
(h)	(232µM)	(1 mM)	(200µM*)	(1mM)	(200µM*)	(200µM)
0.25	39.3	58.5	72.4	——	100	100
0.5	38.9	36.4	——	100	——	100
1.0	28.6	27.7	69.4	100	100	100
2.0	22.9	13.8	47.1	90.7	100	100

* Incubated in 0.1 M instead of 1 M Tris/H_2SO_4 buffer.

The data show that the inactivation is in the order K_2PtCl_4 > trans-platin > cis-platin ≈ $Pt[en]Cl_2$. Also the octahedral complex, hexachloroplatinic acid (H_2PtCl_6) [11] was considerably less active (I_{50} 360 µM after 4 h



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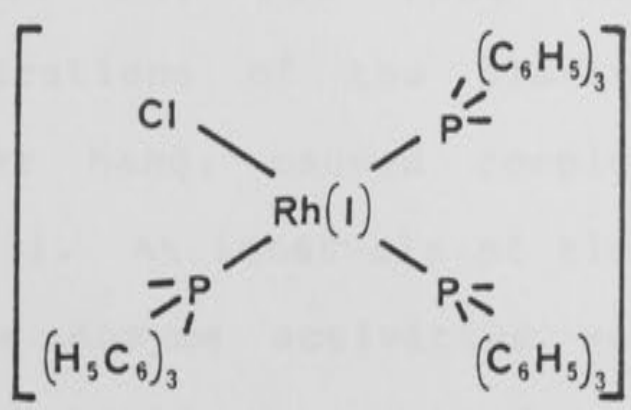


[12]

incubation) than the square planar complex K_2PtCl_4 (I_{50} 20 μM after 15 min incubation) towards human brain DHPR. The rhodium complex, tris(triphenylphosphine)rhodium chloride $[(Ph_3P)_3RhCl]$ [12] which is also geometrically planar was completely inactive. It is known that some rhodium complexes have little anti-tumour activity.[180] These results imply that the inactivation of human brain DHPR by Pt(II) complexes could occur in a multi-dentate manner because K_2PtCl_4 was much more effective than other Pt complexes. On the other hand, in the case of malate dehydrogenase the degree of inhibition by cis-platin and trans-platin was the same,[170] so the Pt(II) complexes may be binding in a mono-dentate manner to this enzyme.

K_2PtCl_4 was found to inactivate human brain DHPR in a time dependent manner. The enzyme and inhibitors were incubated for some time prior to the activity assays. Close to zero time, almost no inactivation was observed but the inhibitory effect increased with longer incubation times.

At lower concentration of K_2PtCl_4 ($PtCl_4^{2-}$) (21 μM) the enzyme activity decreased rapidly during the first hour and then slowed down considerably although the concentration of enzyme (5.6 μM) was less than stoichiometric. Higher concentrations of the platinum complex (210 μM), on the other hand, caused complete inactivation in one hour (Figure 3). At intervals of time, aliquots were dialyzed, but the enzyme activities were found to be unchanged, i.e. partly or completely

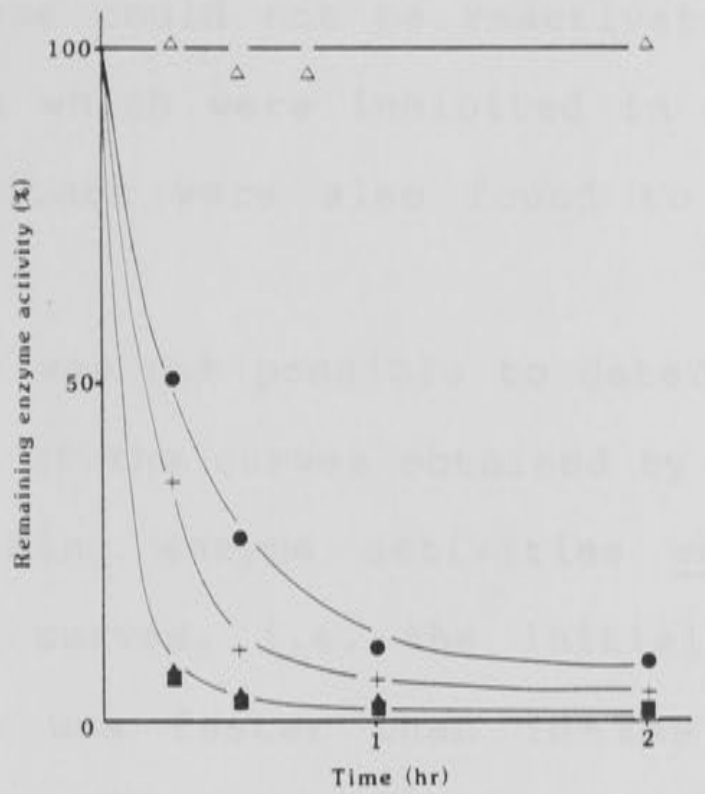


Figure 3. Time dependent inhibition of human brain DHPR by K_2PtCl_4 (20°C, pH 7.2). The concentration of DHPR was 5.6 μM. -△-△- enzyme activity of blank; -●-●- K_2PtCl_4 21 μM; -+-+- 52 μM; -▲-▲- 104 μM; -■-■- 208 μM.

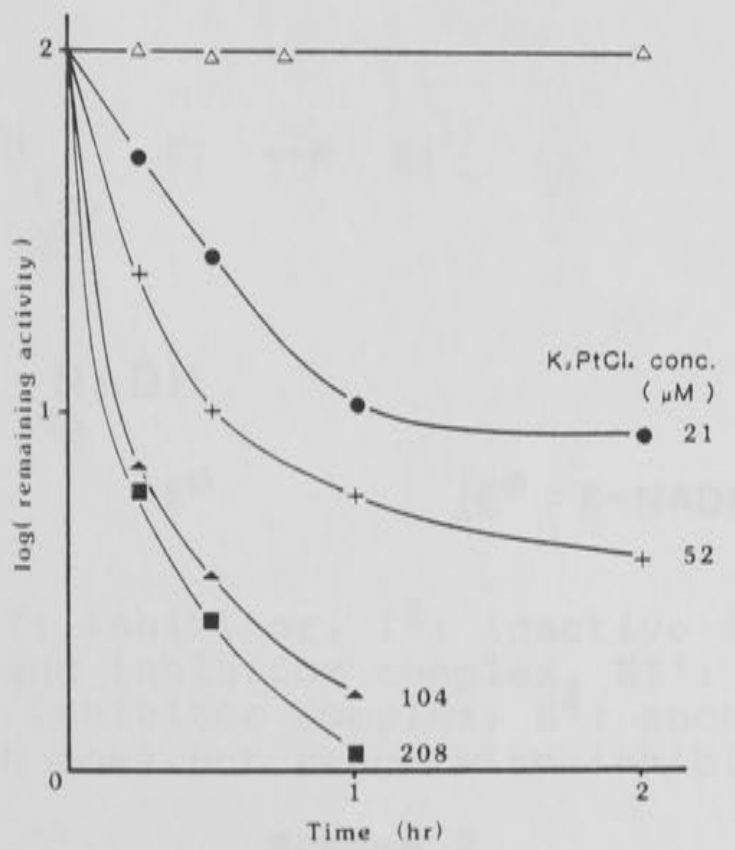
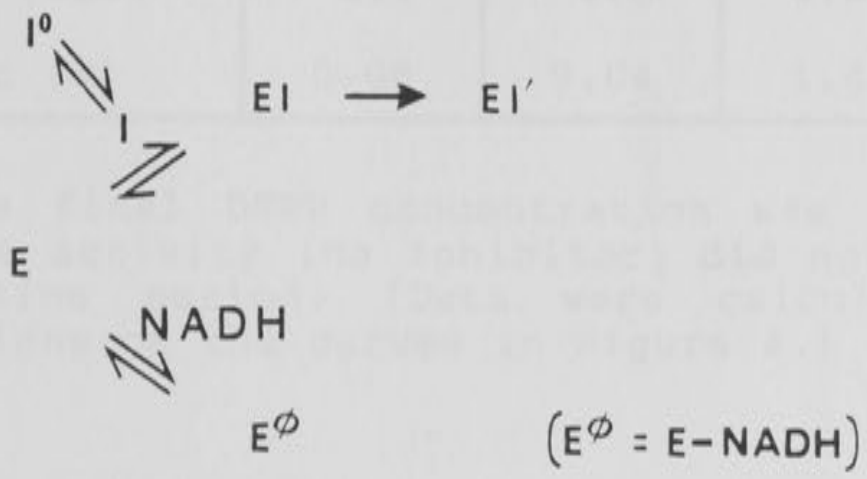


Figure 4. Time dependent inhibition of human brain DHPR by K_2PtCl_4 (20°C, pH 7.2). The values were obtained from Figure 3.

inactivated enzyme could not be reactivated. In general, previous enzymes which were inhibited in a time dependent manner by inhibitors were also found to be irreversibly inhibited.[181]

Here it was not possible to determine a Ki value, because analysis of the curves obtained by plotting the log values of remaining enzyme activities versus incubation times also gave curves, i.e. the initial pre-equilibrium with the enzyme was faster than in the case of malate dehydrogenase and the reactions did not follow simple first order kinetics (Figure 4). The present pattern of plots (Figure 4) are similar to those proposed by Fritzsch[182] for a "two states model" mechanism which is summarized in Scheme 2. In the present examples it is not clear that the



E: enzyme, I: inhibitor, I^0: inactive inhibitor, EI: enzyme and inhibitor complex, EI': inactivated enzyme and inhibitor complex, E^{ϕ}: another form of the enzyme which does not react with inhibitor.

Scheme 2

two species (E and E^{ϕ}) in the mechanism are due to different enzyme species or due to different inhibitor

species. However, the mechanism can be explained by proposing that the inhibitor inactive enzyme species (E^{ϕ}) are in fact the E-NADH complex which has a small dissociation constant (see Chapter 2, Section 2-7, p 65).

Points from the earlier portions of the curves in Figure 3 (p 207) fitted the equation for first order kinetics and gave rough estimates of the early reaction speeds (Table 3).

Table 3. First-order rate constants for the inactivation of DHPR by K_2PtCl_4 at 20^0C.

	K_2PtCl_4 concentration (μM)			
	208	104	52	21
k (h^{-1})	13.3	13.0	6.2	2.5
$t_{1/2}$ (min)	3.1	3.2	6.6	17.0
error (%)	0.08	0.04	1.4	3.0

The final DHPR concentration was 5.6 μM and the blank enzyme activity (no inhibitor) did not change within the incubation period. [Data were calculated from the linear portions of the curves in Figure 4.]

In these measurements, solutions of the complexes were made up in water and incubations of the enzyme with complexes were carried out in 1 M potassium phosphate buffer (pH 7.3).

As shown in Scheme 1 (p 202), an equilibrium is set up between the chloroplatinate and various aquohaloplatinates which are the true inhibitor species.

The inhibitor stock solution is mixed with the enzyme and buffer solution. The equilibria in the stock solutions are set up rapidly (within 5 min) because when the solutions are allowed to age before addition to the enzyme at pH 7.3, no significant differences in the inactivation curves were observed. The reported equilibrium constant[183] for the acid hydrolysis of $PtCl_4^{2-}$ at 25^0C is 3.0×10^{-2} (Equation 2). By using this value, when 208, 104, 52 and 28 μM of K_2PtCl_4 stock solutions were made up in water, they would

$$PtCl_4^{2-} + H_2O \rightleftharpoons PtCl_3(H_2O)^- + Cl^- \quad - - - (2)$$

give mono-aquo species with 6.2, 3.1, 1.6 and 0.6 μM concentrations respectively. The mono-aquo species are also in the equilibrium with diaquo species (Equation 3) and would give concentrations of 0.6, 0.3, 0.15 and 0.06 μM of diaquo species respectively by using 1.0×10^{-1} as equilibrium constant.

$$PtCl_3(H_2O)^- + H_2O \rightleftharpoons PtCl_2(H_2O)_2 + Cl^- \quad - - - (3)$$

The nature of the anion, however, does have an effect on the inhibition curves. Stock solutions of K_2PtCl_4 were made up in 4 mM HBr, 4 mM HCl, 4 mM H_2SO_4 or water and incubated with DHPR in Tris/HBr, Tris/HCl, Tris/H_2SO_4 or phosphate buffers (at pH ~7.3) and assayed at time intervals. The results showed that the degree of inhibition was in the order phosphate > Tris/H_2SO_4 >

Tris/HCl > Tris/HBr buffer (Table 4).

Table 4. The effect of buffer anion on the inactivation of DHPR by K_2PtCl_4 at 20°C.

DHPR (5.6 μM) was incubated with inhibitor (200 μM) in each buffer solution and aliquots (1 μl) were assayed in the respective buffer.

Time (h)	Remaining enzyme activity (%)			
	Tris/HBr	Tris/HCl	Tris/H_2SO_4	Phosphate
0.25	28.7	17.9	13.4	4.8
0.5	12.6	8.5	8.9	2.5
1.0	6.8	4.3	3.7	0
2.0	——	——	2.0	0

This is different from the result which was obtained by Teggins et al.[171,173] on the inhibition of malate dehydrogenase. They demonstrated that bromide ligands were more labile than chloride ligands in substitution reaction of Pt(II) complexes.[173]

The concentration of buffer was also found to affect the rate of inactivation of DHPR. Incubation at pH 7.3 with 0.03 M Tris/H_2SO_4 or 0.03 M phosphate buffer caused more rapid inactivation of DHPR than the corresponding buffers at 0.3 M concentrations with same enzyme and $PtCl_4^{2-}$ concentrations (Table 5).

Table 5. The effect of different buffer concentrations on the inactivation of DHPR by K_2PtCl_4 at 20°C.

DHPR (5.6 µM) was incubated with K_2PtCl_4 (230 µM in 4 mM H_2SO_4) in each buffer and aliquots (1 µl) were assayed.

Time (h)	Remaining enzyme activity (%)			
	Tris/H_2SO_4		Phosphate	
	0.03 M	0.3 M	0.03 M	0.3 M
0.25	13.4	45.1	4.8	10.4
0.5	8.9	39.0	2.5	5.9
1.0	3.7	——	0	2.7
2.0	2.0	33.3	0	1.2
12.5	0	22.9	0	0.9

Attempts to remove all the chloride from $PtCl_4^{2-}$ by reaction with silver nitrate caused immediate inhibition of DHPR, but this is most probably due to traces of silver which is a potent inhibitor of enzymes.[184] The complete inhibition by 4 µM silver nitrate of human brain DHPR was almost instantaneous (this work).

Dhondt and Bellahsene[95] reported that inhibition of DHPR from rat tissues by cis-platin was partially protected by increasing Cl^- ions in the solutions. The present study confirms this observation. When stock solutions of K_2PtCl_4 were made in 0.3 M KCl and in 3.0 M KCl and then incubated with human brain DHPR in 0.1 M phosphate buffer (pH 7.3), i.e. final concentrations were

0.1 M and 1 M KCl, the rate of inhibition in the solution made from the latter stock solution was slower (Table 6).

Table 6. The effect of KCl on DHPR inactivation by K_2PtCl_4 at 20°C, pH 7.2.

Time (h)	Remaining enzyme activity (%)		
	H_2O	0.1 M KCl	1 M KCl
0.25	6.0	28.8	43.6
0.5	2.6	8.6	43.6
1.0	1.1	6.4	13.2
2.0	1.3	2.6	9.1

These data support the contention that more of $PtCl_4^{2-}$ complex species are present in the 1 M KCl solution than the other solutions during the incubation and that they are less reactive towards the enzyme than the other platinum species in the equilibria in Scheme 1 (p 202).

The statement was made earlier (p 208) that the inactivation process involves a pre-equilibrium step followed by faster irreversible inactivation of the enzyme. The Pt(II) complex bound enzyme does not dissociate easily because when the solutions used for the data in Figure 3 (p 207), except the blank solutions, were dialyzed, the original enzyme activities were not recovered.

Good evidence that the platinum complex reacted at or near the active site of DHPR was that the activity was

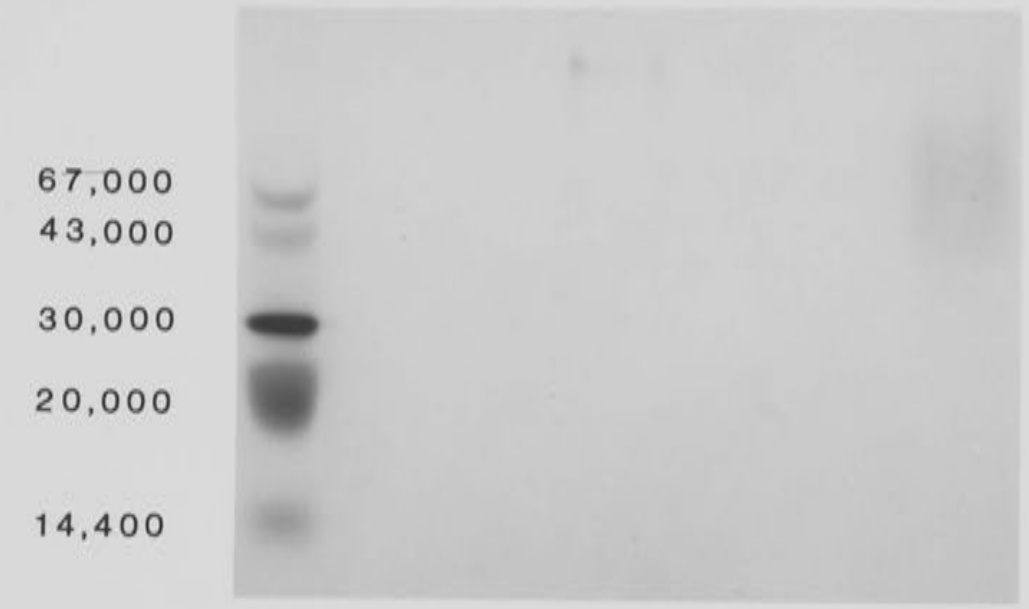
completely protected when NADH at 114 μM was pre-incubated with the enzyme (2.6 μM) and then mixed with K_2PtCl_4 at 204 μM. When the enzyme was pre-incubated with K_2PtCl_4 and then NADH was added at intervals of time, immediate protection occurred (i.e. no further time dependent inactivation), but the original activity of the enzyme was not recovered. These could be explained by Scheme 2 (p 208), because in the earlier time of the incubation there was still some free enzyme (i.e. not bound to $PtCl_4^{2-}$), and NADH can protect the free enzyme but not replace the bound Pt(II) in the enzyme complex readily. Thus the platinum complex at or near the active site cannot be reversibly removed by NADH, but NADH at the active site will inhibit the reaction of K_2PtCl_4 at this site. On the other hand, NAD^+ at 152 μM provided no protection from inactivation by K_2PtCl_4. Also incubation of DHPR with K_2PtCl_4 (233 μM) in the absence of NADH but in the presence of 2.7 mM DTT afforded complete protection. This, however, is attributed to the formation of stable Pt(II)-DTT complexes (DTT is in large excess) which react very slowly (or not at all) with the enzyme and implies that $PtCl_4^{2-}$ reacts with thiol groups and consequently with a thiol group(s) at the active site (see Section 4-3-5, p 222). Because NADH protected DHPR, the enzyme used in all the above measurements was exhaustively dialyzed to remove bound NADH.

4-4-4 Gel electrophoresis, UV and fluorescence studies of the enzyme inactivated by potassium tetrachloroplatinate

Polyacrylamide gel electrophoresis of the $PtCl_4^{2-}$-inactivated human brain DHPR, after dialysis at 4^0C under non-denaturing conditions to remove free $PtCl_4^{2-}$, gave a diffused band at high molecular weight similar to that observed for the non-denatured DHPR (Figure 5, A). This suggests that the Pt-enzyme complex remained in a dimeric form like the active enzyme and also $PtCl_4^{2-}$ is binding to the enzyme subunit without intermolecular (oligomer) binding (see below). It should be noted that Firgaira et al.[10] had found that on PAGE human liver DHPR gave a band at higher Mr values (the Mr values depended on gel concentrations,) than for the dimer (Mr ca 50,000) due to a low net charge to size ratio for this protein.

Electrophoresis of the inhibited enzyme in the presence of SDS (denaturing conditions), on the other hand, gave a single monomer band at a slightly higher Mr value (27,000) than the uncomplexed monomer enzyme (26,000) (Figure 5, B) indicating that the platinum may well be still bound to the enzyme. This could not be confirmed by elemental analysis because large amounts of enzyme were required for this purpose. Human brain DHPR always shows two very close bands on SDS-PAGE when mercaptoethanol is excluded (probably due to the presence of 'oxidized' enzyme), whereas the platinum inactivated enzyme gives only a simple band (cf. Chapter 2, Section 2-2, p 38). It must

A) PAGE



B) SDS-PAGE

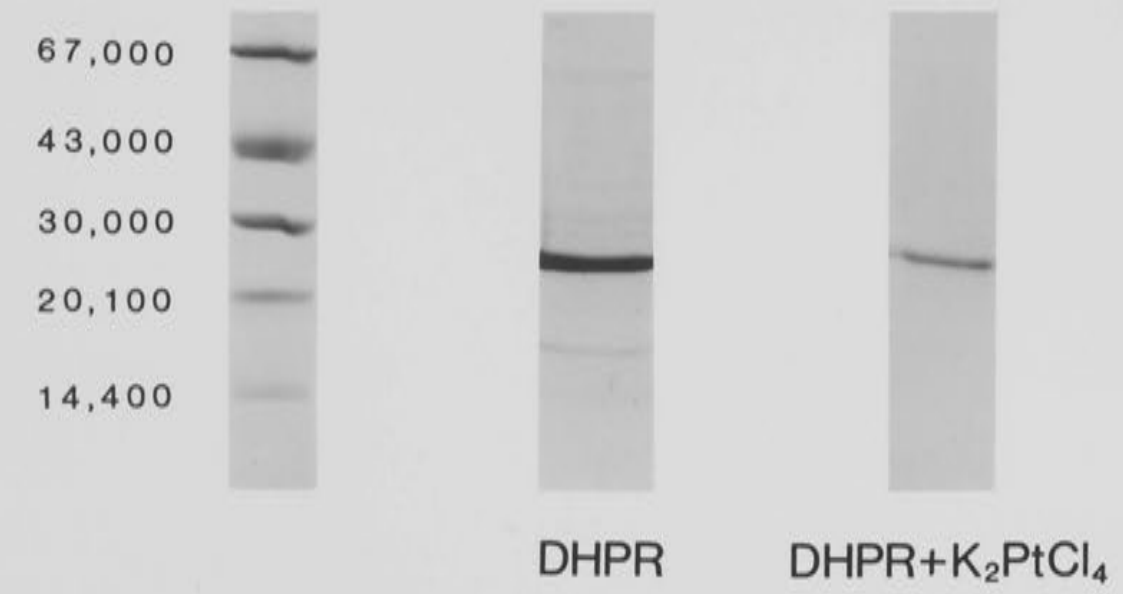


Figure 5. Gel electrophoresis of K_2PtCl_4 inactivated DHPR.

be pointed out that platinum complex inactivated DHPR is not stained by Commassie Blue as readily as active DHPR and larger amounts of enzyme were required for staining.

When a solution of DHPR (DTT free) was incubated with K_2PtCl_4 in potassium phosphate buffer (pH 7.2) at 25^0C, the UV maximum at 280 nm moved to shorter wavelength with time and at complete inactivation (5 h) it was at ca 270 nm (Figure 6). This may be indicative of conformational changes due to the reaction of the enzyme with K_2PtCl_4.

In order to study this further the fluorescence spectra of this system were examined. The intensity of the fluorescence maximum at 350 nm (excitation at 280 nm) of DHPR was found to decrease with time after addition of K_2PtCl_4 (one molecular equivalent) with a small bathochromic shift of 3 nm (Figure 7). The rate of decrease of intensity increased with increasing K_2PtCl_4 concentration. No change in intensity was observed when K_2PtCl_4 was added to DHPR which had been denatured with 4M urea for 17 h, (4 M urea had weak fluorescence with λ_{max} at 364 nm due to impurities), also the emission maximum had not changed from 357 nm which was the emission maximum for urea denatured DHPR (Figure 8). On comparing the rate of inactivation of DHPR with the rate of decrease in the intensity of the band at 350 nm it was found that the former rate was ca 2.4 times faster than the rate of quenching, and quenching was still proceeding after the enzyme had been completely inactivated (Figure 9). This

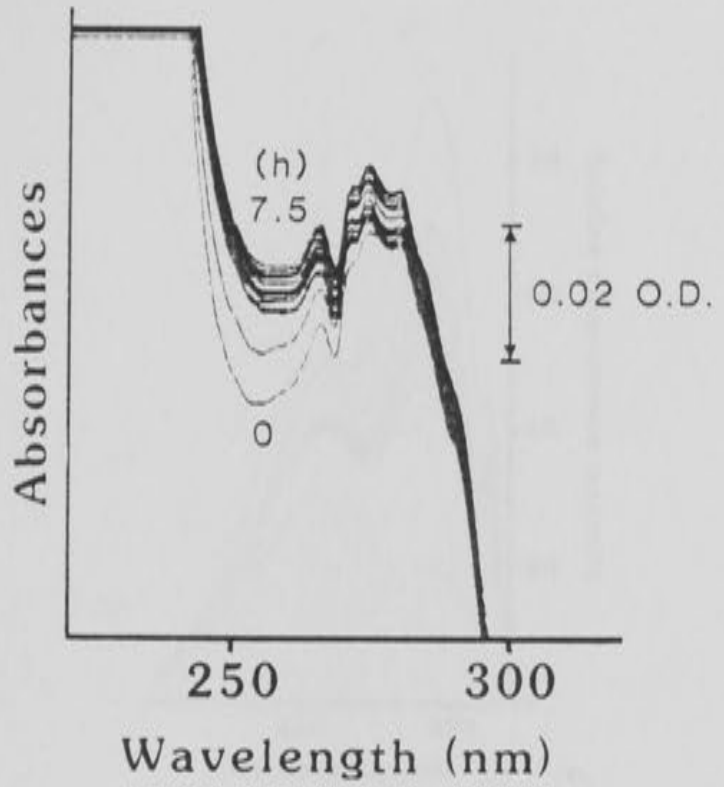


Figure 6. UV spectral changes of potassium tetrachloroplatinate inactivated DHPR. DHPR (1.3 μM) was incubated with K_2PtCl_4 (107 μM) in 50 mM phosphate buffer (pH 7.2) at 25°C. The spectra were scanned at 30 min intervals.

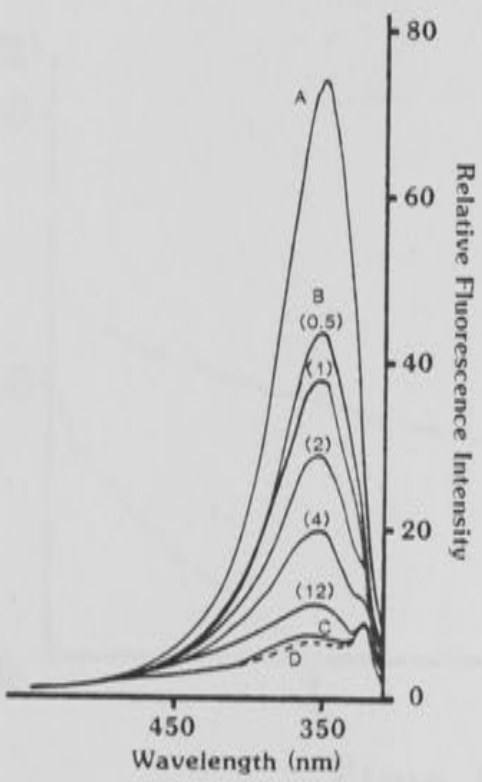


Figure 7. Fluorescence emission spectral changes obtained from incubation of human brain DHPR and K_2PtCl_4 at 20°C. A: DHPR (0.34 μM) in 0.1 M phosphate buffer (pH 7.2); B: DHPR (0.31 μM) and K_2PtCl_4 (93.6 μM) in the same buffer, times after mixing are in parentheses (h); C: K_2PtCl_4 (93.5 μM); D: buffer.

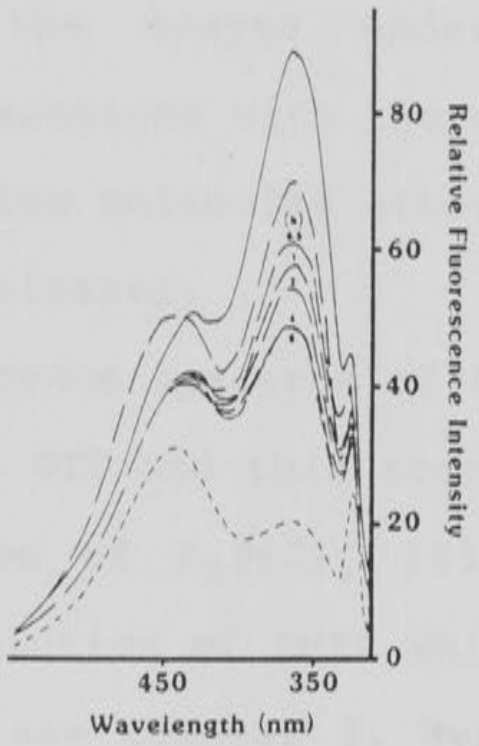


Figure 8. Fluorescence emission spectra of urea denatured DHPR with K_2PtCl_4 at 20°C. —— DHPR in 4 M urea (pH 7.2), incubated for 17 h; – – addition of K_2PtCl_4, times after mixing (h); - - - 4 M urea in phosphate buffer (pH 7.2).

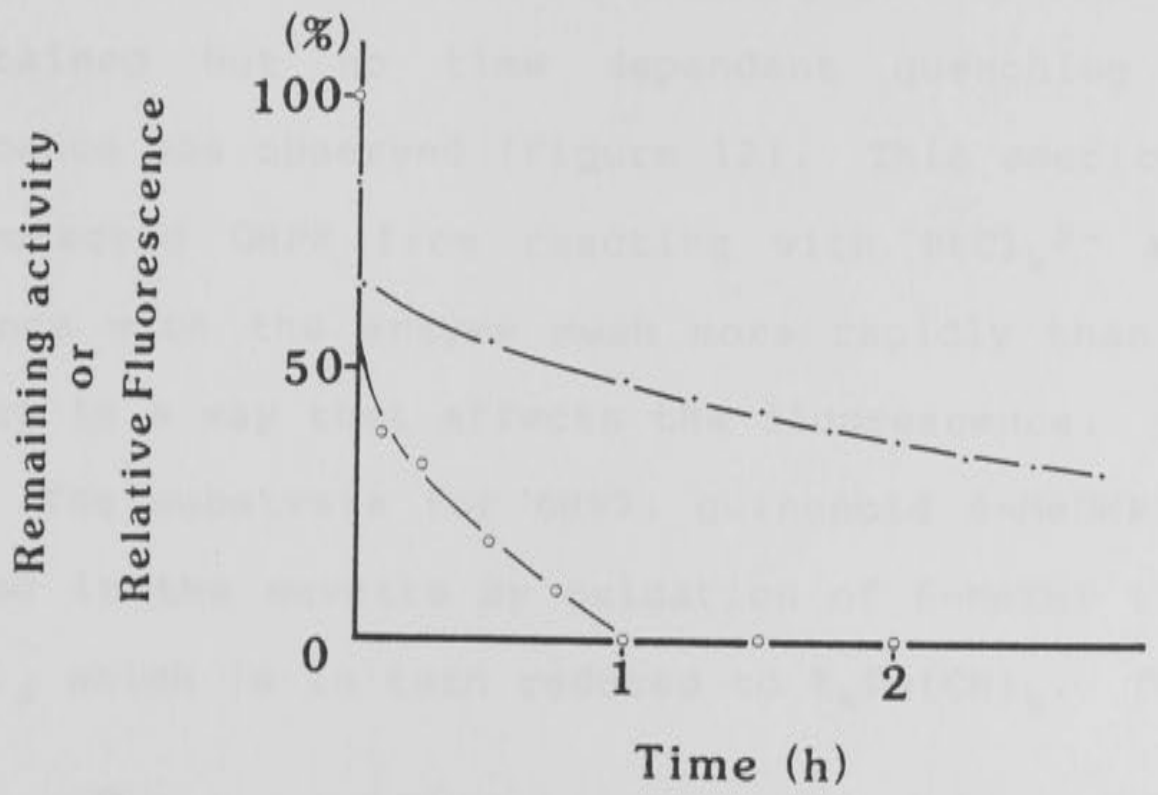
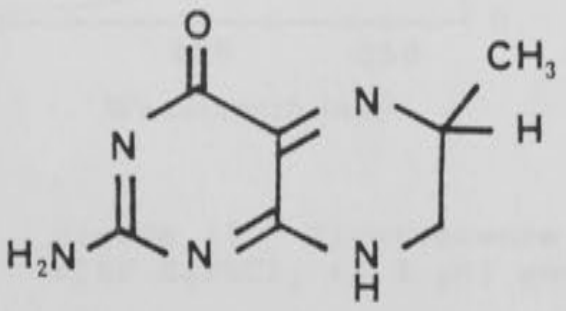


Figure 9. The rates of fluorescence changes and enzyme activities. DHPR (0.25 μM) was incubated with K_2PtCl_4 (93.6 μM) and the fluorescence emission spectra and enzyme activities were measured at time intervals. -o-o- remaining enzyme activity (%); -+-+- relative fluorescence intensity (%).

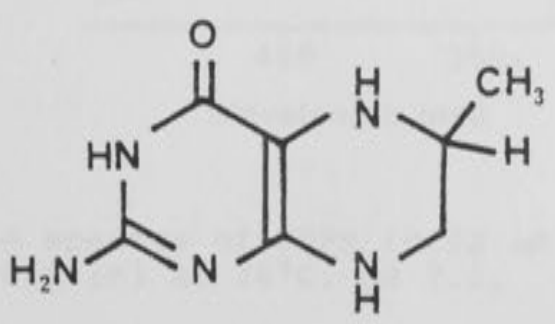
demonstrates that the enzyme undergoes conformational changes by further reactions with the same (intramolecular) or other Pt(II) complex molecules after the active site had been completely inactivated.

The fluorescence spectrum of DHPR was unaltered in the presence of 2 mM DTT and this spectrum was then almost unchanged on addition of K_2PtCl_4 (85.4 µM) (Figure 10). The spectrum of a solution of DHPR which was pre-incubated with NADH (0.9 µM) (see Chapter 2, Section 2-5, p 47) was unaltered when K_2PtCl_4 (9.5 µM) was added (Figure 11-A). Also when DHPR was mixed with K_2PtCl_4, the decrease in fluorescence intensity with time at 350 nm could be immediately halted by addition of NADH (Figure 11-B). Moreover, when a mixture of NADH and K_2PtCl_4 was added to a solution of DHPR the normal quenching due to NADH binding was obtained but no time dependent quenching of the fluorescence was observed (Figure 12). This confirmed that NADH protected DHPR from reacting with $PtCl_4^{2-}$ and that NADH binds with the enzyme much more rapidly than $PtCl_4^{2-}$ can react in a way that affects the fluorescence.

The substrate for DHPR, quinonoid 6-MeDHP [13] is generated in the cuvette by oxidation of 6-MeTHP [14] with $K_3Fe(CN)_6$ which is in turn reduced to $K_4Fe(CN)_6$. These two



[13]



[14]

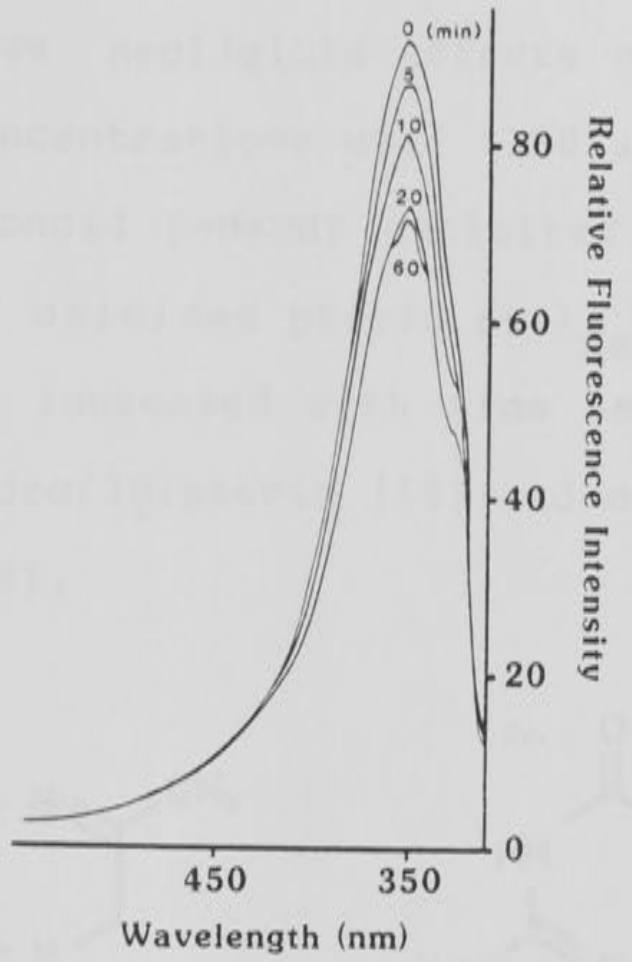


Figure 10. Fluorescence emission spectra of DHPR (0.14 µM) with K_2PtCl_4 (85.4 µM) and DTT (2 mM) at 20°C.

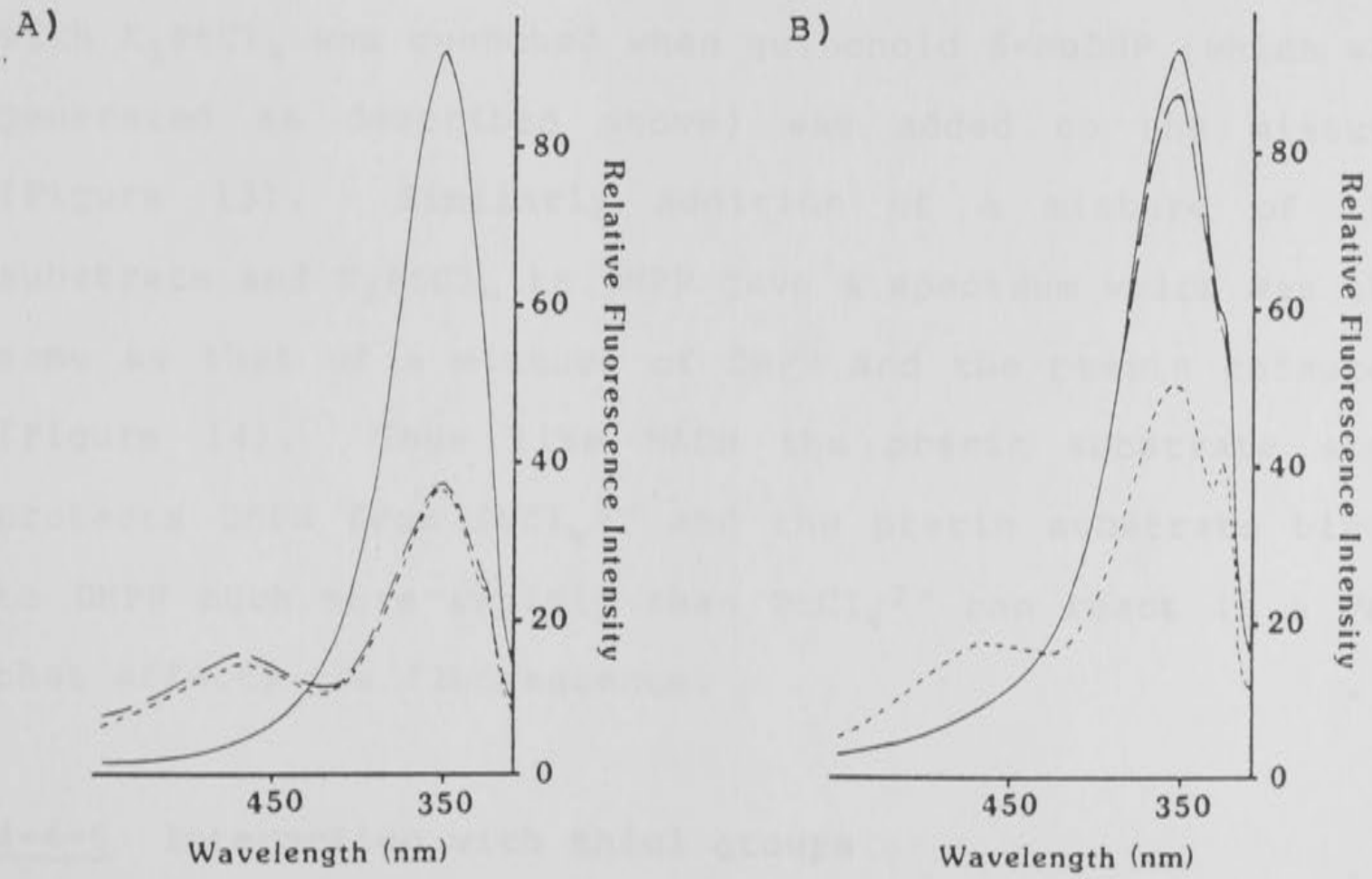
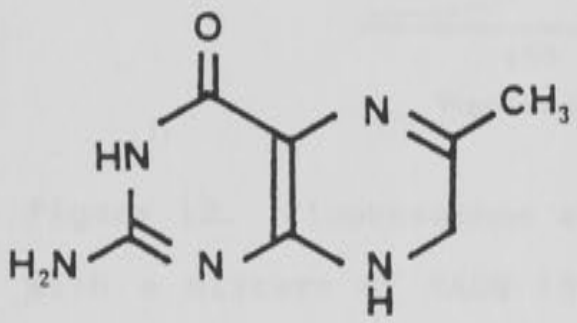


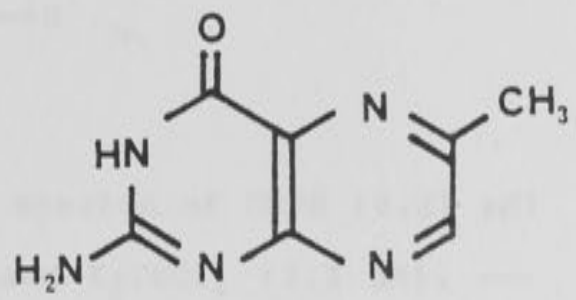
Figure 11. Fluorescence emission spectra of DHPR (0.32 µM) with K_2PtCl_4 (2.3 µM) and NADH (0.8 µM) at 20°C, pH 7.2.

A) —— DHPR; – – NADH was added to DHPR; - - - K_2PtCl_4 was added to the mixture of NADH and DHPR.
B) —— DHPR; – – DHPR and K_2PtCl_4; - - - NADH was added to the mixture of DHPR and K_2PtCl_4.

iron complexes have negligible affects on the fluorescence of DHPR at the concentrations used (100 µM) (see Section 4-3, p 193). Quinonoid 6-MeDHP exhibited some fluorescence due to traces of oxidized pterin at λ_{max} 445 nm (emission wavelength) which increased with time as it rearranged to 6-methyl-7,8-dihydro(3H)pterin [15] and oxidized further to 6-methylpterin [16].



[15]



[16]

The fluorescence emission at 350 nm of DHPR pre-incubated with K_2PtCl_4 was quenched when quinonoid 6-MeDHP (which was generated as described above) was added to the mixture (Figure 13). Similarly addition of a mixture of the substrate and K_2PtCl_4 to DHPR gave a spectrum which was the same as that of a mixture of DHPR and the pterin cofactor (Figure 14). Thus like NADH the pterin substrate also protects DHPR from $PtCl_4^{2-}$ and the pterin substrate binds to DHPR much more rapidly than $PtCl_4^{2-}$ can react in a way that affects the fluorescence.

4-4-5 Interaction with thiol groups

The above data imply that $PtCl_4^{2-}$ reacts primarily with some amino acid(s) at or near the active site of DHPR

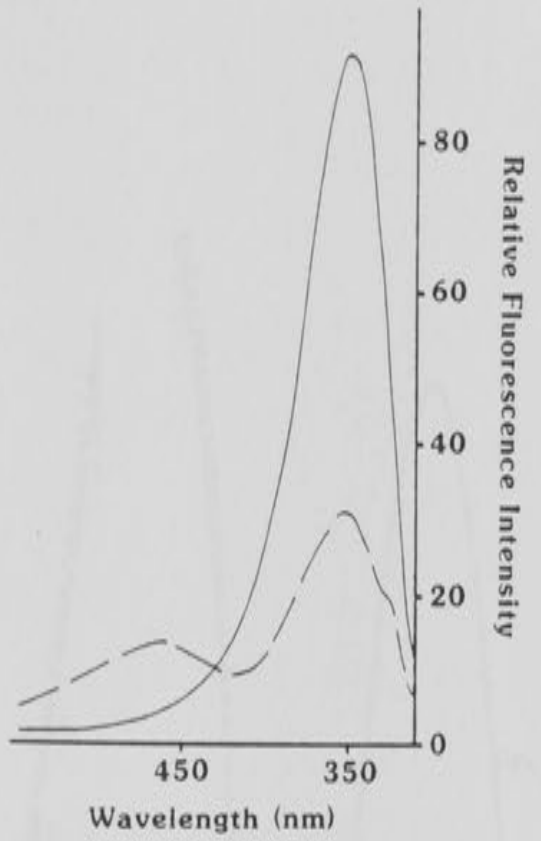


Figure 12. Fluorescence emission spectra of DHPR (0.31 µM) with a mixture of NADH (0.8 µM) and K_2PtCl_4 (2.2 µM). —— DHPR; – – addition of the mixture.

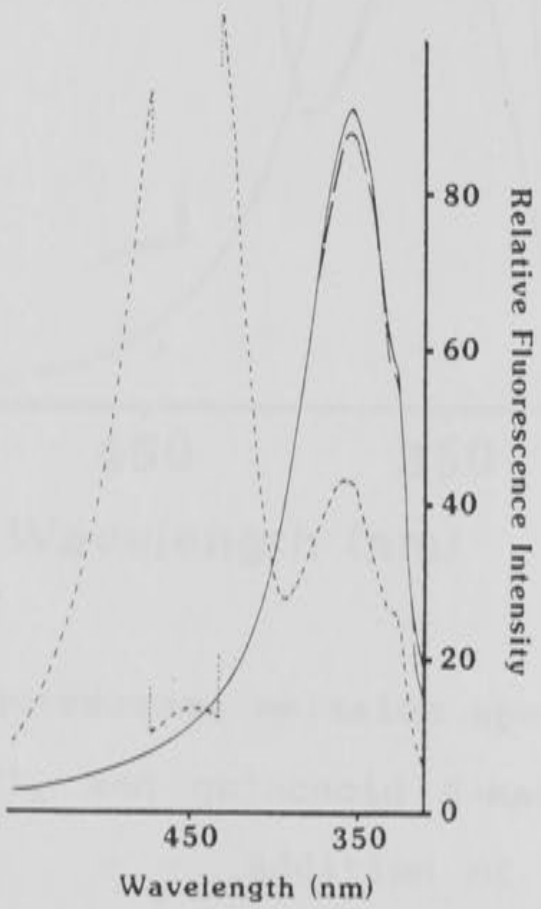


Figure 13. Fluorescence emission spectra of DHPR (0.31 µM) with K_2PtCl_4 (2.3 µM) and quinonoid 6-MeDHP (43 µM). —— DHPR in phosphate buffer (pH 7.2); – – K_2PtCl_4 was added to DHPR; - - - 2 min after, quinonoid 6-MeDHP was added to the mixture of DHPR and K_2PtCl_4.

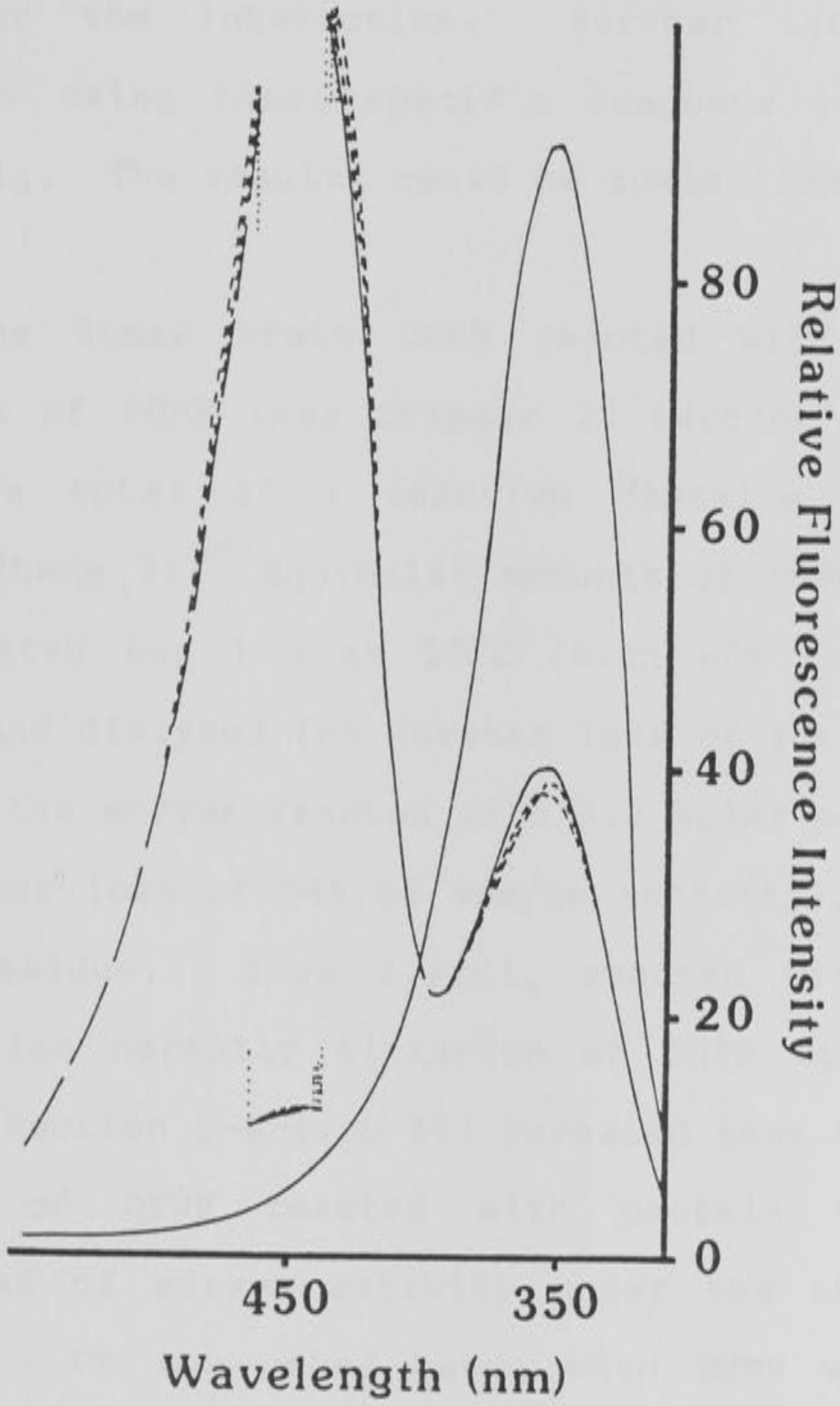


Figure 14. Fluorescence emission spectra of DHPR with a mixture of K_2PtCl_4 and quinonoid 6-MeDHP. —— DHPR in buffer (pH 7.2); – – addition of the mixture; - - - oxidation of quinonoid 6-MeDHP.

with covalent binding. The following results strongly support the contention that thiol groups in the protein are involved in the interaction. Further information was obtained by using thiol specific reagents in conjunction with K_2PtCl_4. The results could be summarized as in Scheme 3 (p 227).

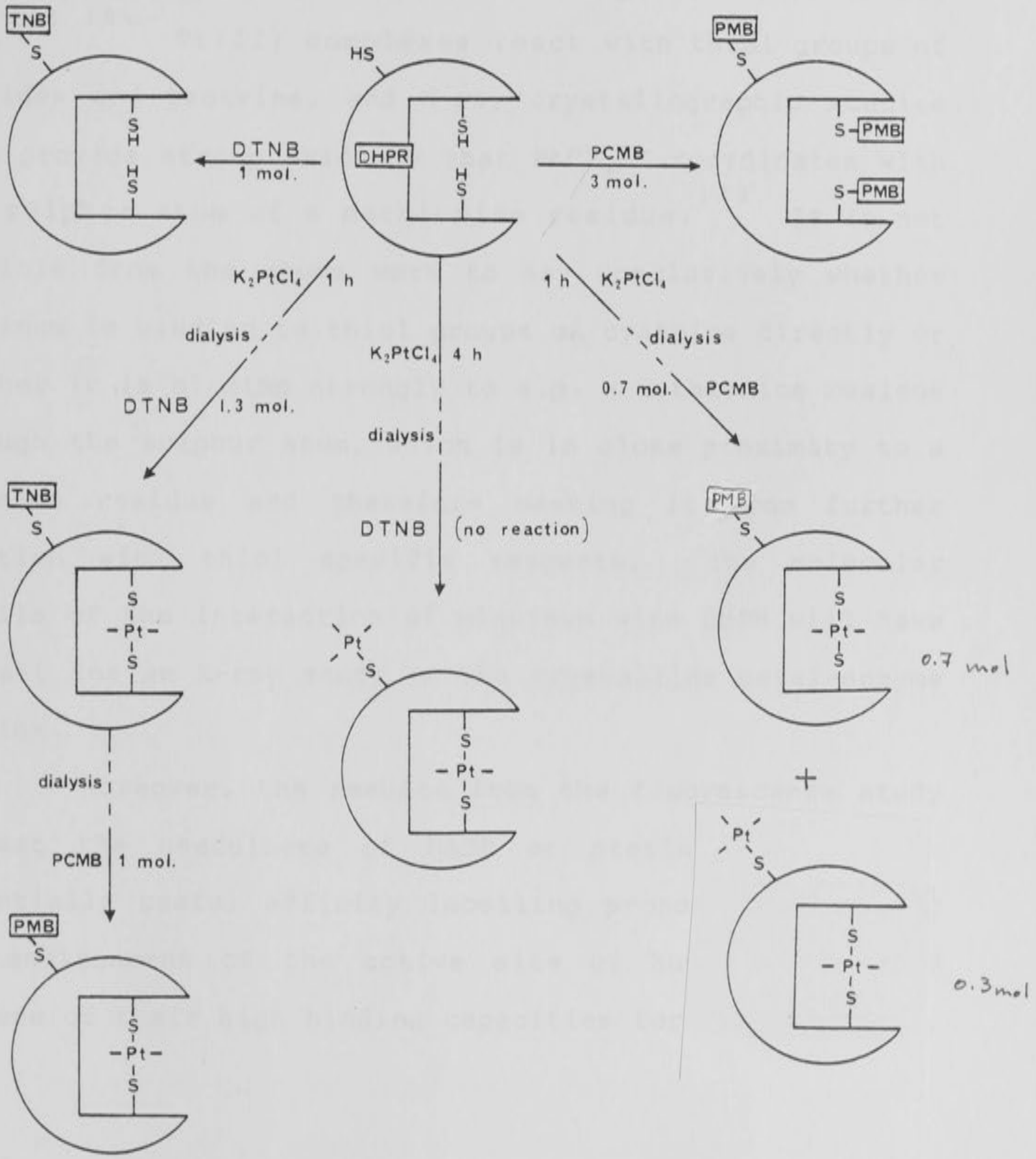
The human brain DHPR reacted with 3 molecular equivalents of PCMB (see Chapter 2, Section 2-6-2, p 60) inferring a total of 3 reactive cysteine residues per subunit (Scheme 3). Equimolar amounts of DHPR and K_2PtCl_4 were incubated for 1 h at 20^0C (with 60% loss of enzyme activity) and dialyzed (no further loss of activity). Upon titration, the enzyme reacted with 0.7 molar equivalents of PCMB (further loss of 24% of enzyme activity), i.e. ca one cysteine residue. Thus K_2PtCl_4 reacted with two thiol groups. Also normally titration of DHPR with DTNB (see Chapter 2, Section 2-6-1, p 57) revealed that one molecular equivalent of DTNB reacted with protein thiol groups without loss of enzyme activity under the non-denaturing conditions. On the other hand, when DHPR was incubated with an equimolar amount of K_2PtCl_4 (1 h, 60% loss of enzyme activity) followed by dialysis; then titration with DTNB showed that 1.3 equivalents (ca one -SH) of the latter reacted. If this solution was dialyzed again, the enzyme then consumed one molecular equivalent of PCMB with 32% loss of enzyme activity. When the original incubation time of DHPR and K_2PtCl_4 was lengthened to 4 h, no thiol group was titrated by DTNB. These data are consistent with

$PtCl_4^{2-}$ reacting with more than one thiol group of the enzyme (possibly two -SH groups). Moreover, $PtCl_4^{2-}$ binds to (or masks) (i.e. at or near the active site) two internal (buried) thiol groups first and then later binds to (or masks) one cysteine residue which is located far from the first two thiol groups or a second Pt(II) molecule reacts with the enzyme (Scheme 3).

The further loss of 24% of enzyme activity by reacting with one molar equivalent of PCMB (Scheme 3) could be explained by further conformational changes of the enzyme caused by this binding, or PCMB exchanged one of bindings between Pt(II) and enzyme which was not yet stabilized. It can be concluded that when the reaction between Pt(II) complex and enzyme is complete, more than one Pt(II) molecule per subunit have reacted because it is unlikely that all -SH groups are in such proximity to the enzyme as to form tri-dentate binding on to one platinum molecule.

4-5 Conclusion

Among several inhibitors studied K_2PtCl_4 showed the strongest inactivating effect on human brain DHPR by reacting in at last a bi-dentate manner after a long incubation period. In an incubation period it appears that the stoichiometry is one Pt per subunit (cf. this is in good agreement with the result from SDS-PAGE, p 215) but after long incubation the stoichiometry is at least two Pt



Scheme 3

DHPR DHPR subunit; TNB thionitrobenzoate; PMB p-mercuribenzoate.

per subunit. The coordination of compounds of elements related to platinum (e.g. mercury, silver and lead) with thiol groups on the protein and causing inhibition is not new.[161,184] Pt(II) complexes react with thiol groups of peptides and proteins, and X-ray crystallographic studies also provide strong evidence that $PtCl_4^{2-}$ coordinates with the sulphur atom of a methionine residue.[175] It is not possible from the above work to say conclusively whether platinum is binding to thiol groups of cysteine directly or whether it is binding strongly to e.g. a methionine residue through the sulphur atom, which is in close proximity to a cysteine residue and therefore masking it from further reaction with thiol specific reagents. The molecular details of the interaction of platinum with DHPR will have to wait for an X-ray study of the crystalline metal-enzyme complex.

Moreover, the results from the fluorescence study suggest the usefulness of NADH or pterin analogues as potentially useful affinity labelling probes for studying the environment of the active site of human brain DHPR because of their high binding capacities for this enzyme.

4-6 Experimental

4-6-1 Materials and methods

A: Materials

All commercial chemicals were of the highest commercially available purity.

K_2PtCl_4, cis-platin, trans-platin were from Aldrich Chemical Co., Milwaukee, USA. H_2PtCl_6, $(Ph_3P)_3RhCl$ were purchased from Fluka AG, Buchs, Switzerland. $Pt[en]Cl_2$ was prepared as before.[185] 6-MeTHP and 6,7-Me_2THP hydrochlorides were kindly supplied by Dr W.L.F. Armarego and pteridines were synthesized in this work (cf. Chapter 3).

Human brain DHPR used in this chapter was purified for this work and the details were described in Chapter 2, Section 2-2 (p 38), and then dialyzed against 50 mM or 0.1 M potassium phosphate buffer (pH 7.2)

B: Methods

Ultraviolet spectra, kinetic assays and PCMB titrations were performed on Unicam SP 1800 or Cary 219 double beam spectrometers. The determination of concentrations at λ_{max} and DTNB titrations were carried out on a Perkin-Elmer Lambda 1 single beam spectrometer.

Fluorescence spectra were measured as described before (cf. Chapter 2, Section 2-9-1, p 72).

The protein concentration was determined by the

Bio-Rad microassay (cf. Chapter 2, Section 2-9-5, p 81).

Gel electrophoresis was performed as before (cf. Chapter 2, Section 2-9-1, p 72).

PCMB and DTNB titrations were also performed as before (cf. Chapter 2, Section 2-9-8, p 83).

The incubations of inhibitor and the enzyme were carried out in clean, capped glass vials.

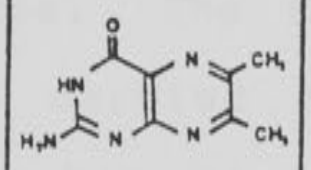
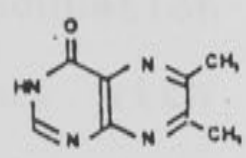
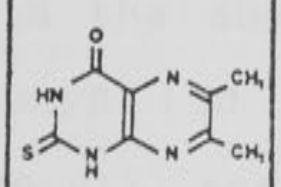
4-6-2 Inhibition by oxidized pteridines

i) Human brain DHPR (10 µl in 0.1 M potassium phosphate buffer, 5.6 µM), 0.1 M phosphate buffer (pH 7.2) (10 µl) and inhibitor solution (10 µl in 4 mM HCl) were incubated at 20^0C. The blank incubation was carried out by using 4 mM HCl (10 ml) instead of the inhibitors. Pteridin-4(3H)-one (206 µM) and 6,7-dimethylpteridin-4(3H)-one (185 µM) solutions (in 4 mM HCl) were used as inhibitors. After 3 or 30 min, aliquots (1 µl) were assayed with phosphate buffer (100 µl, 0.1 M), NADH (25 µl in 50 mM Tris/HCl buffer, 100 µM), 6-MeTHP (15 µl in 4 mM HCl, 20 µM) and water (810 µl) at 30^0C. The reaction was initiated by addition of 2 mM $K_3Fe(CN)_6$ (50 µl, 100 µM) and the initial rates of changes of the absorbance at 340 nm (ε 6,200 $M^{-1}.cm^{-1}$ for NADH) were measured. The blank cuvette did not contain the enzyme solution. Both the 3 and 30 min incubations showed no inhibitor activity by these pteridines. If, as described in the literature,[17] these were reversible inhibitors, the long incubation period was

not necessary. So it was concluded that these pteridines were not inhibitors for human brain DHPR.

ii) The inhibition of sheep liver DHPR which was kindly supplied from Dr W.L.F. Armarego, was carried out in the same way as in the literature[17] (i.e. no previous incubation). The assay solutions contained inhibitor solution (50 µl), phosphate buffer (pH 7.2) (100 µl, 0.1 M), NADH (25 µl, 100 µM), 6,7-Me_2THP (40 µl, 20 µM) and water (735 µl, 30 ^{0}C). Sheep liver DHPR (5 µl) was added only to the sample cuvette and the reaction was initiated by addition of 2 mM $K_3Fe(CN)_6$ (50 µl, 100 µM) to both cuvettes (Table 7). The blank experiment was carried out with 4 mM HCl (50 µl) instead of inhibitor solution.

Table 7 Inhibition of sheep liver DHPR with pteridinones at 30^{0}C, pH 7.2

Inhibitor							blank
concn (µM)	89.4	447.0	55.3	110.6	106.0	211	
remaining activity (%)	100	50	100	100	100	80	100

4-6-3 Effects of potassium ferricyanide on DHPR activity

Incubation of DHPR and $K_3Fe(CN)_6$ were carried out at 20^{0}C in the following solutions; DHPR (DTT free) (10 µl, 3.6 µM), 0.1 M potassium phosphate buffer (pH 7.2) (10 µl) and 92.2 mM (or 60.3 or 30.3 mM) $K_3Fe(CN)_6$ (10 µl, final concentrations in incubation mixtures were respectively 30.7, 20.1 or 10.1 mM). Aliquots (2 µl) of these solutions were taken at time intervals and mixed with the assay cocktail [potassium phosphate buffer (pH 7.2) (100 µl, 0.1 M), NADH (25 µl, 96 µM), 6-MeTHP (15 µl, 22.2 µM) and water (810 µl)] in the sample cuvette at 25^{0}C. The blank incubation solution contained 50 mM potassium phosphate buffer (pH 7.2) (10 µl), 0.1 M phosphate buffer (pH 7.2) (10 µl) and $K_3Fe(CN)_6$ (10 µl, final concentrations were respectively 30.7, 20.1 or 10.1 mM) and kept at 20^{0}C, and the aliquots were taken to the blank cuvette. The reaction was initiated by addition of 2 mM $K_3Fe(CN)_6$ (50 µl, 100 µM) to both cuvettes. The incubation in the absence of the $K_3Fe(CN)_6$ was carried out with DHPR (10 µl), 0.1 M phosphate buffer (10 µl) and water (10 µl), and aliquots (2 µl) were assayed as above.

No inhibition of DHPR activity was noted even after 3 h incubation.

4-6-4 Inactivation by potassium ferrocyanide

The incubations were carried out in the following solution; DHPR (DTT free) (10 µl, 3.6 µM), 0.1 M phosphate

buffer (pH 7.2) (10 µl) and $K_4Fe(CN)_6$ (10 µl, final concentrations were 10.1, 20.2 and 30.2 mM) at 20°C. Aliquots (2 µl) were added to the mixture of phosphate buffer (pH 7.2) (100 µl, 0.1 M), NADH (25 µl, 86 µM), 6-MeTHP (15 µl, 23 µM) and water (810 µl, 25°C). The reaction was initiated by addition of 2 mM $K_3Fe(CN)_6$ (50 µl, 100 µM). The blank incubation was carried out with water instead of inhibitor solution. The results are shown in Table 8 and Figure 1 (p 195).

Table 8. Inactivation of human brain DHPR with potassium ferrocyanide at 20°C, pH 7.2.

Time (h)	Remaining activity (%)						
	$K_4Fe(CN)_6$ (mM)						
	0	10.1	20.2				30.2
				a	b	c	
0.08	——	85.8	46.7	——	——	——	41.0
0.25	——	——	18.3	57.6	——	100	16.9
0.5	100	34.7	16.4	——	84.0	93.8	13.0
1.0	100	20.2	14.0	17.6	63.5	78.4	14.0
2.0	100	12.0	11.6	15.5	31.0	70.0	12.5
3.0	100	11.6	12.5	——	21.0	75.0	11.1
6.0	100	14.9	14.0	——	——	——	14.9

a: incubated with 2.0 mM dithiothreitol
b: incubated with 5.5 µM NADH
c: incubated with 100 µM NADH

iii) DHPR (DTT free) (10 µl, 3.6 µM) was incubated with dithiothreitol (10 µl in 0.1 M phosphate buffer, 4.0 mM) for 10 min at 18°C and then $K_4Fe(CN)_6$ (10 µl, 20.4 mM) was added to it (final DTT concentration was 2.0 mM). Aliquots (2 µl) were assayed as above (Table 8).

iv) DHPR (DTT free) (10 µl) was incubated with NADH [10 µl in 0.1 M phosphate buffer (pH 7.2), 8.3 or 150 µM] for 10 min at 18^0C and then $K_4Fe(CN)_6$ was added to the mixture (10 µl, 20.4 mM) (final NADH concentration was 5.5 or 100 µM). Aliquots (2 µl) were taken at intervals of time and then assayed as above. The blank experiment was carried out with water (10 µl) instead of inhibitor solution (Table 8 and Figure 1, p 195).

v) Attempts were made to determine the Ki value of this inhibitor. The enzyme and inhibitor solutions were incubated as above (ii) for 5 min and the enzyme activity was assayed at various concentrations of 6-MeTHP. The results were analyzed with a computer programme,[159] but the results did not fit into the known inhibition patterns properly, i.e. the errors of the Ki values were not acceptable. The plot of the log values of remaining activities against incubation times showed curved lines. In general to obtain kinetic parmeters, this plot should be a straight line, i.e. reversible reaction.

vi) DHPR (DTT free) (10 µl, 0.3 µM) was incubated with 5.2 mM $K_4Fe(CN)_6$ (10 µl, 100 µM) and 0.1 M phosphate buffer (pH 7.2) (490 µl) and the fluorescence emission spectra (slit 5 nm) were scanned with time at 20^0C. The excitation wavelength was 280 nm (slit 5 nm) and λ_{max} of this mixture was 355 nm, and the fluorescence intensity at this wavelength decreased by 3% in 10 min. After 10 min, NADH

(10 µl, 4 µM) was added to it and the solution was scanned again (Figure 2, p 197). The quenching of DHPR due to NADH binding (cf. Chapter 2, Section 2-5, p 47) was now not so strong.

4-6-5 Inactivation by Pt(II) complexes

i) Pt(II) complexes (10 µl in 4 mM H_2SO_4) were incubated with DHPR (60 µM DTT) (10 µl, 5.3 µM) in Tris/H_2SO_4 buffer (pH 7.2) (10 µl, 0.3 M) at 20°C. Aliquots (1 µl) were assayed with Tris/H_2SO_4 buffer (pH 7.2) (100 µl, 0.1 M), NADH (25 µl, 110 µM), 6-MeTHP (15 µl, 23 µM) and water (810 µl, 25°C) and the reaction was initiated by the addition of $K_3Fe(CN)_6$ (50 µl, 100 µM). The blank cuvette did not contain incubation aliquots and the changes of absorbance at 340 nm were measured at 25°C. The blank experiment was carried out by using 4 mM H_2SO_4 (10 µl) instead of inhibitor solution and the activity did not alter during 2 h (Table 2, p 205 and Table 9). The Pt(II) complexes were K_2PtCl_4, cis-platin, trans-platin, $Pt[en]Cl_2$, H_2PtCl_6 and rhodium complex, $(Ph_3P)_3RhCl$ (this complex required heating to dissolve).

Table 9. Inactivation of DHPR by metal complexes.

Time (h)	Remaining activity (%)	
	H_2PtCl_6 (360 µM)	$(Ph_3P)_3RhCl$ (~300 µM)
0.08	100	100
0.17	83	100
1.0		100
4.0	50	100

4-6-6 Inactivation by potassium tetrachloroplatinate

Time dependent inactivation of human brain DHPR: All stock solutions of K_2PtCl_4 were freshly prepared prior to each experiment. K_2PtCl_4 (10 µl in water) was incubated with DHPR (10 µl, 5.6 µM) in potassium phosphate buffer (pH 7.2) (10 µl, 0.1 M) at 20^0C. Aliquots (1 µl) were sampled at time intervals and mixed with phosphate buffer (100 µl, 0.1 M), NADH (25 µl, 110 µM), 6-MeTHP (15 µl, 23 µM) and water (810 µl, 25^0C). The assay was initiated by addition of 2 mM $K_3Fe(CN)_6$ (50 µl, 100 µM) to both cuvettes. The blank experiment was carried out using water (10 µl) instead of the inhibitor solution (Tables 10, 11 and Figures 3, 4, p 207).

After 2 h, the incubation mixtures were dialyzed in small dialysis tubes against 4 µM NADH in 50 mM phosphate buffer (pH 7.2) for 24 h at 4^0C and no recovery of enzyme activity was observed (including dilution factor on dialysis).

Table 10. Time dependent inactivation of DHPR by K_2PtCl_4 at 20^0C.

Time (h)	Remaining activity (%)				
	K_2PtCl_4 (µM)				
	0	20.8	52	104	208
0.25	100	50.3	24.1	7.2	6.0
0.5	100	26.7	10.1	3.5	2.6
0.75	100	21.0	9.4	——	——
1.0	100	10.5	5.9	2.6	1.1
1.5	100	10.1	4.8	——	0.9
2.0	100	8.8	3.9	0.4	1.3

Table 11. Time dependent inactivation of DHPR by K_2PtCl_4 at 20^{0}C.

Time (h)	log [remaining activity (%)]			
	K_2PtCl_4 (µM)			
	20.8	52	104	208
0.25	1.70	1.38	0.86	0.78
0.5	1.43	1.00	0.54	0.41
0.75	1.32	0.97	———	———
1.0	1.02	0.77	0.41	0.04
1.5	1.00	0.68	———	-0.05
2.0	0.94	0.59	-0.40	0.11

First order rate constants for the earlier part of curves in Figure 3 were calculated by using computer programme which was kindly supplied by Dr D. Randles. The results were shown in Table 3 (p 209).

Effect of anion: K_2PtCl_4 stock solutions (10 µl, 200 µM) were made in 4 mM HBr, 4 mM HCl, 4 mM H_2SO_4 or H_2O and incubated with DHPR (DTT free) (10 µl, 5.6 µM) in 10 µl of 0.1 M Tris/HBr (pH 7.3), 0.1 M Tris/HCl (pH 7.3), 0.1 M Tris/H_2SO_4 (pH 7.3) or 0.1 M potassium phosphate (pH 7.2) buffer respectively at 20^{0}C and aliquots (1 µl) were assayed as above in the respective buffer (Table 4, p 211).

Effect of buffer concentration: K_2PtCl_4 stock solution (10 µl in 4 mM H_2SO_4) was incubated with DHPR (DTT free) (10 µl, 5.6 µM) in 10 µl of Tris/H_2SO_4 buffer (pH 7.3) (0.03 or 0.3 M) or potassium phosphate buffer (pH 7.2) (0.03 or 0.3 M) at 20^{0}C and aliquots (1 µl) were assayed as

above in the respective buffer (0.1 M). The blank activity (no inhibitor) was unchanged during 12.5 h (Table 5, p 212).

Effect of Cl^- concentration:

i) K_2PtCl_4 stock solution (10 µl, 210 µM) was dissolved in water, 0.3 M KCl in water or 3.0 M KCl in water and incubated with DHPR (DTT free) (10 µl, 5.6 µM) in potassium phosphate buffer (pH 7.2) (10 µl, 0.3 M). Aliquots (1 µl) were assayed as above in 0.1 M phosphate buffer (Table 6, p 213).

ii) K_2PtCl_4 (2.49 mg, 6.0 µmol) was dissolved in water (10 ml) and silver nitrate (4.08 mg, 24 µmol) was added to it and stirred at 25^0C, overnight. The precipitate was removed by centrifugation and the supernatant was used for the incubation. This solution [10 µl, expected as 200 µM of $Pt(H_2O)_4^{2+}$] was incubated with DHPR (10 µl, 5.6 µM) in 0.1 M phosphate buffer (pH 7.2) (10 µl) and aliquots (1 µl) were assayed at time intervals as above. Enzyme activity was already lost after 15min.

When dilute HCl was added to this supernatant, the solution immediately became turbid, indicating that Ag^+ ions were present in the solution. Silver is a well known enzyme inhibitor which binds to thiol groups and this might be the true inhibitor in this experiment.

iii) Silver nitrate stock solution (10 µl in water, final

concentrations in the incubation were 4, 400 and 800 µM) was incubated with DHPR (10 µl, 5.6 µM) in 0.1 M phosphate buffer (10 µl) at 20°C. Aliquots were taken after 5, 10 and 30 min and assayed. No remaining enzyme activity was observed even with 4 µM silver nitrate and 5 min incubation period.

Protection with NADH , dithiothreitol or NAD^+:

i) DHPR (10 µl, 2.6 µM) was pre-incubated with NADH (7 µl in 50 mM Tris/HCl buffer, 114 or 266 µM) in 0.1 M Tris/HCl buffer (pH 7.3) (8 µl) for 10 min at 20°C. The stock solution of K_2PtCl_4 (5 µl in 4 mM HCl, 102 or 204 µM) was added to the above incubation mixture and aliquots (2 µl) were assayed as before at time itervals. The blank incubation was carried out without inhibitor. No loss of the enzyme activity was observed.

ii) DHPR (10 µl, 2.6 µM) was pre-incubated with K_2PtCl_4 (10 µl in 4 mM HCl, 204 µM) in 0.1 M Tris/HCl buffer (pH 7.3) (10 µl) for 1.25 h at 20°C and then NADH (7 µl, 242 µM) was added. Aliquots (2 µl) were assayed at time intervals but no further time dependent loss of enzyme activity was observed, although the orginal enzyme activity could not be recovered within the 11.5 h incubation period.

iii) Dithiothreitol (5 µl, 4 mM) was pre-incubated with K_2PtCl_4 (10 µl in 4 mM HCl, 350 µM) in 0.1 M Tris/HCl

buffer (pH 7.3) (5 µl) for 3 h at 20°C and then DHPR (10 µl, 2.6 µM) was added to the mixture (final concentrations of DTT and K_2PtCl_4 were 2.7 mM and 233 µM respectively) and aliquots (2 µl) were assayed as before at time intervals. The blank incubation was carried out with 4 mM HCl (10 µl) instead of the inhibitor solution. Enzyme activity did not change within a 2 h incubation period.

The pre-incubation was also carried out with DHPR (10 µl) and DTT (5 µl, 2.7 mM) in 0.1 M Tris/HCl buffer (pH 7.3) (5 µl) for 30 min and then K_2PtCl_4 (5 µl, 350 µM) was added and aliquots (2 µl) were assayed (Table 12).

Table 12. Protection by DTT (2.7 mM).

Time (min)	Remaining activity (%)	
	K_2PtCl_4 (233 µM)	Blank
2	100	100
7	75	100
15	64	—
30	64	100
45	64	100

iv) DHPR (10 µl, 2.6 µM) was pre-incubated with NAD^+ (5 µl) in 0.1 M Tris/HCl buffer (pH 7.3) (10 µl) for 10 min at 20°C and then K_2PtCl_4 (10 µl in 4 mM HCl, 102 µM) was added (final concentrations of NAD^+ were 38.0 and 151.9 µM). Aliquots (2 µl) were assayed at time intervals. The blank incubations were carried out with 4 mM HCl (10 µl) at two different NAD^+ concentrations or in the absence of NAD^+ solution (Table 13).

Table 13. The effect of NAD^+ on the inactivation of DHPR (2.6 µM) by K_2PtCl_4 (102 µM).

Time (h)	Remaining activity (%)				
	NAD^+ (µM)				
	0*	38.0	151.9	Blank#	
				38.0	151.9
0.25	7.2	61.2	52.3	100	100
0.5	3.5	——	30.0	100	100
1.0	2.6	32.9	20.4	100	100
2.0	0.4	——	——	100	100
10.0	——	25.5	16.0	88.5	100

* Contains K_2PtCl_4 (104 µM) but no NAD^+ in solution.

\# No K_2PtCl_4 in solution.

Gel electrophoreses:

i) DHPR (20 µl) was incubated with K_2PtCl_4 (20 µl in water, 220 µM) in 0.1 M phosphate buffer (pH 7.2) (20 µl) for 2 h at 25^{0}C and no remaining enzyme activity was observed. This solution was dialyzed against 0.1 M phosphate buffer (pH 7.2) for 4.5 h at 4^{0}C (250 ml x 3) but no enzyme activity was recovered. Aliquots were applied on a gel for PAGE (7.5% running gel) (Figure 5, p 216). The inhibited DHPR always showed a diffuse band around the dimer position as with native DHPR.

ii) DHPR (40 µl) was incubated with K_2PtCl_4 (40 µl in water, 220 µM) in 0.1 M phosphate buffer (pH 7.2) (40 µl) for 1 h at 25^{0}C and then the mixture was dialyzed against 0.1 M phosphate buffer (pH 7.2) (200 ml x 3) for 2 h at 4^{0}C. Aliquots (20 µl) were mixed with 10% SDS (10 µl) and β-mercaptoethanol (1 drop), and incubated for 3 min at 100^{0}C and sucrose solution (to make up 10% concentration) was added and then applied on a gel for SDS-PAGE (15% running gel) (Figure 5, p 216). The blank sample was prepared by mixing DHPR (10 µl), 10% SDS (5 µl) and β-mercaptoethanol (1 drop), and incubated for 3 min at 100^{0}C prior to applying onto gel.

UV spectra:

i) DHPR (DTT free) (50 µl in 0.1 M phosphate buffer, 1.3 µM) was incubated with K_2PtCl_4 (50 µl in water, 107 µM) in 50 mM phosphate buffer (pH 7.2) (200 µl) for 7.5 h at

25^0C. The blank cell contained 0.1 M phosphate buffer (50 µl) instead of enzyme solution. The spectra were scanned every 30 min with a Cary 219 double beam spectrometer (Figure 6, p 218).

Fluorescence spectra:

i) DHPR (10 µl, 0.31 µM) was incubated with K_2PtCl_4 (50 µl in water, 93.6 µM) in 0.1 M phosphate buffer (pH 7.2) (490 µl) at 20^0C and the fluorescence emission spectra were scanned from 310 to 550 nm (slit 5 nm). The fluorescence due to the protein decreased with time (Figure 7, p 218).

ii) DHPR (5 µl) was incubated with 4 M urea in 0.1 M phosphate buffer (pH 7.6) (595 µl) at 20^0C and the fluorescence emission spectra were scanned for 13 h. In this incubation the fluorescence intensity decreased with time, but after 7 h to 13 h it did not change further (61.3% of original intensity), and λ_{max} was finally at 357 nm whereas λ_{max} was at 352 nm prior to addition of urea. After 17 h, K_2PtCl_4 solution (50 µl, 79.2 µM) was added to the above solution but the fluorescence intensity and λ_{max} did not change after a further 21 h incubation period (Figure 8, p 219).

iii) The rate of inactivation of DHPR and the rate of decrease in fluorescence intensity at λ_{max} were compared by incubating DHPR (10 µl, 0.25 µM) with K_2PtCl_4 (50 µl, 93.6 µM) in 0.1 M phosphate buffer (pH 7.2) (490 µl) in the

fluorescence spectrometer cuvette at 20^0C. The fluorescence intensity at 350 nm was measured at time intervals and aliquots (5 µl) were simultaneously assayed for enzyme activity as before (Figure 9, p 219).

iv) DHPR (5 µl, 0.14 µM) was incubated with 2 mM DTT in 0.1 M phosphate buffer (pH 7.2) (595 µl) at 20^0C and K_2PtCl_4 (50 µl, 85.4 µM) was added, and then the fluorescence emission spectra were scanned (Figure 10, p 221). The spectral changes were slower than the one in Figure 7 (p 218) in which the protein concentration was higher (0.31 µM) than in this case.

v) DHPR (10 µl, 0.32 µM) was pre-incubated with NADH (10 µl, 0.9 µM) in 0.1 M phosphate buffer (pH 7.2) (490 µl) at 20^0C and the fluorescence emission spectra were scanned (no changes within 15 min after quenching due to NADH binding). K_2PtCl_4 (50 µl, 9.5 µM) was added to the above solution but the fluorescence emission spectra were not changed (Figure 11-A, p 221).

vi) DHPR (10 µl, 0.32 µM) was pre-incubated with K_2PtCl_4 (10 µl, 2.3 µM) in 0.1 M phosphate buffer (pH 7.2) (490 µl) at 20^0C and the fluorescence emisssion spectra were scanned. NADH (10 µl, 0.8 µM) was added to it and the fluorescence emission spectra showed quenching due to NADH binding but no time dependent decrease of fluorescence (Figure 11-B, p 221).

vii) DHPR (10 µl, 0.31 µM) was dissolved in 0.1 M phosphate buffer (pH 7.2) (490 µl) and then a mixture of NADH (10 µl, 0.8 µM) and K_2PtCl_4 (10 µl, 2.2 µM) was added to the enzyme solution. The fluorescence spectra were scanned at 20^0C and the spectra were immediately quenched but no time dependent fluorescence change was observed (Figure 12, p 223).

viii) DHPR (10 µl, 0.31 µM) was pre-incubated with K_2PtCl_4 (10 µl, 2.3 µM) in 0.1 M phosphate buffer (pH 7.2) (490 µl) at 20^0C and the fluorescence emission spectra showed time dependent changes. Quinonoid 6-MeDHP, which was generated by mixing of 6-MeTHP (10 µl, 43 µM) and $K_3Fe(CN)_6$ (10 µl, 100 µM), was added to the above mixture and the fluorescence spectra showed quenching due to the substrate binding on to the protein (Figure 13, p 223).

ix) DHPR (10 µl, 0.31 µM) was dissolved into 0.1 M phosphate buffer (pH 7.2) (490 µl) and then the mixture of K_2PtCl_4 (10 µl, 189 µM) and 6-MeTHP (10 µl, 43 µM) and $K_3Fe(CN)_6$ (10 µl, 100 µM) was added to the enzyme solution. The fluorescence emission spectra showed quenching due to substrate binding but no time dependent decrease in fluorescence occurred (Figure 14, p 224).

4-6-7 Interaction with thiol groups

i) DHPR (100 µl, 1.92 nmol) was incubated with K_2PtCl_4 (19 µl, 1.9 nmol) in 0.1 M phosphate buffer (pH 7.2) (181

µl) for 1 h at 20^{0}C and aliquots (1 µl) were assayed at time intervals. 60 percent of the original enzyme activity was lost within 1 h. The enzyme solution was dialyzed against 50 mM phosphate buffer (pH 7.2) (250 ml x 4) for 14 h at 4^{0}C and during the dialysis no change of enzyme activity was observed. Aliquots were mixed with 50 mM phosphate buffer (60 µl) and titrated with PCMB. The blank cuvette contained 300 µl of 50 mM phosphate buffer (pH 7.2) and also aliquots (3 µl) were assayed. 0.7 molecular equivalents of PCMB had reacted with 24% loss of enzyme activity (Figure 15).

ii) DHPR (100 µl, 1.92 nmol) was incubated with K_2PtCl_4 (19 µl, 1.9 nmol) in 0.1 M phosphate buffer (pH 7.2) (181 µl) for 1 h at 21^{0}C and aliquots (1 µl) were assayed (60% loss of the original enzyme activity). This solution was dialyzed against 0.1 M phosphate buffer (pH 7.2) (200 ml x 2) for 8.5 h at 4^{0}C (no enzyme activity change was observed) and aliquots (200 µl) were mixed with 0.1 M phosphate buffer (pH 7.2) (100 µl) and 10 mM DTNB solution (30 µl, 300 nmol). 1.3 equivalents of DTNB reacted with enzyme thiol groups in a 3 h incubation period. This solution was again dialyzed against 0.1 M phosphate buffer (pH 7.2) (200 ml x 2) for 9 h at 4^{0}C and aliquots (250 µl) were mixed with 0.1 M phosphate buffer (pH 7.2) (50 µl) and titrated with PCMB at 25^{0}C ($\lambda_{analyt.}$ 250 nm). 1.0 equivalent of PCMB reacted with further 32% loss of the enzyme activity.

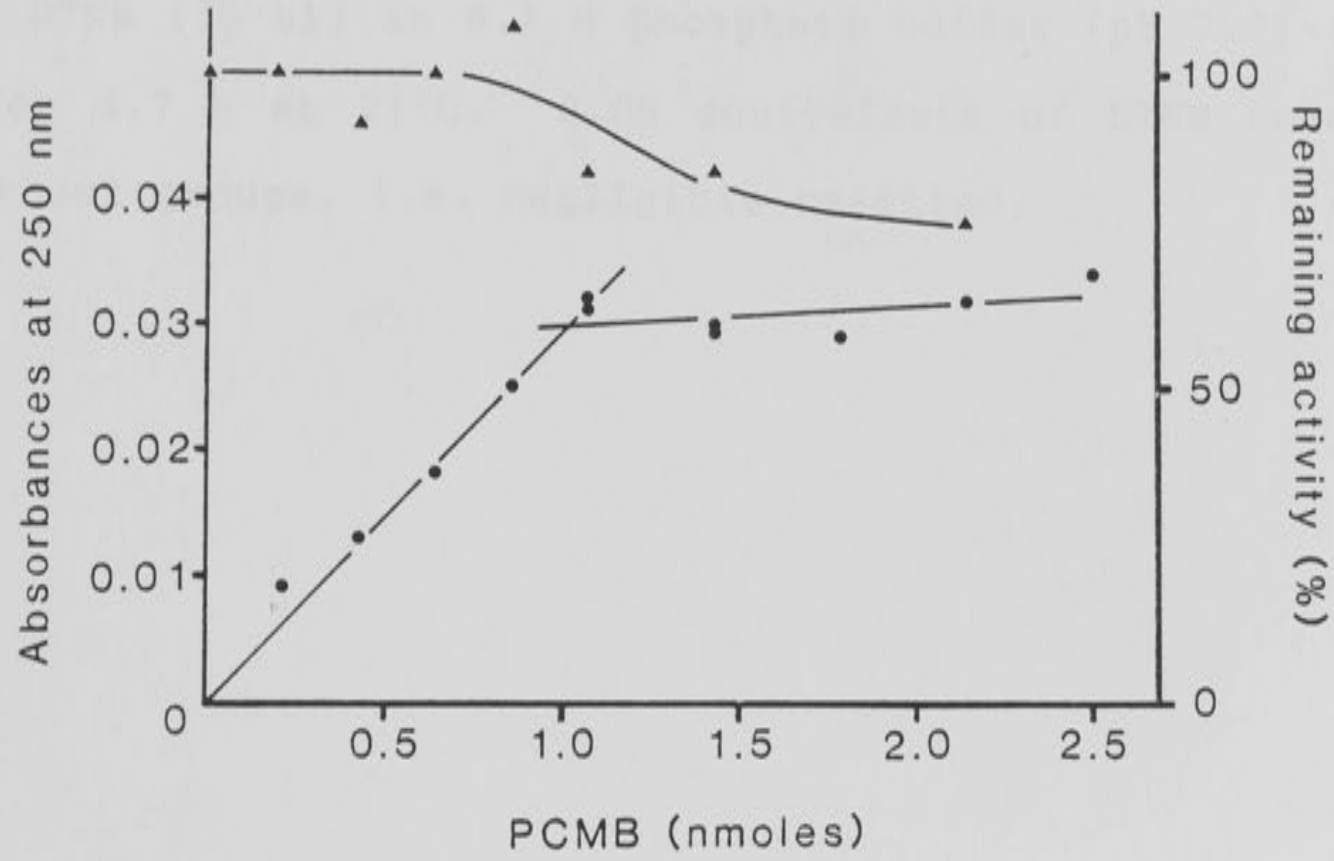


Figure 15. PCMB titration curve of (DHPR-$PtCl_4^{2-}$) complex.

DHPR concentration was 1.46 nmoles in the titration cuvette. -●-●- absorbance changes at 250 nm; -▲-▲- enzyme activity.

iii) DHPR (100 µl, 1.92 nmol) was incubated with K_2PtCl_4 (19 µl, 1.9 nmol) in 0.1 M phosphate buffer (pH 7.2) (181 µl) for 4 h at 21^{0}C (60% loss of enzyme activity) and then dialyzed against 0.1 M phosphate buffer (pH 7.2) (200 ml x 2) for 15 h at 4^{0}C. Aliquots (200 µl) were incubated with 10 mM DTNB (30 µl) in 0.1 M phosphate buffer (pH 7.2) (100 µl) for 6.7 h at 21^{0}C. 0.09 equivalents of DTNB reacted with thiol groups, i.e. negligible reaction.

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APPENDIX

Publications

Wilfred L.F. Armarego, Akiko Ohnishi and Hiroyasu Taguchi

"New pteridine Substrates for Dihydropteridine Reductase and Horseradish Peroxidase" (1986) Biochem. J. **234** 335-342

Wilfred L.F. Armarego, Akiko Ohnishi and Hiroyasu Taguchi

"Reduced 2-Methyl-4-oxo, 2-Methylamino-4-oxo, 2-Methylthio-4-oxo and 2-Amino-4-thioxo 6,7-Dimethylpteridines, and 6,8-Dimethylpterin as Substrates for Dihydropteridine Reductase" (1986) Aust. J. Chem. **39** 31-41

Wilfred L.F. Armarego and Akiko Ohnishi

"Inactivation of Dihydropteridine Reductase (Human Brain) by Platinum(II) Complexes" (1987) Eur. J. Biochem. (accepted, 16th December, 1986)

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New pteridine substrates for dihydropteridine reductase and horseradish peroxidase

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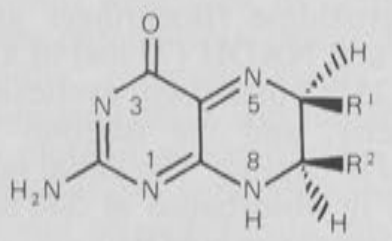
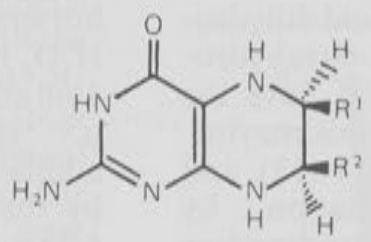
The oxidation of 4,5-diaminopyrimidin-6(1*H*)-one, 5,6,7,8-tetrahydropteridin-4(3*H*)-one, its 6-methyl and *cis*-6,7-dimethyl derivatives, and 6-methyl- and *cis*-6-7-dimethyl-5,6,7,8-tetrahydropterins, by horseradish peroxidase/H_2O_2 is enzymic and follows Michaelis–Menten kinetics, and its K_m and $k_{cat.}$ values were determined. This oxidation of 5,6,7,8-tetrahydropterins produces quinonoid dihydropterins of established structure, and they are known to be specific substrates for dihydropteridine reductase. By analogy the peroxidase/H_2O_2 oxidation of the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones should produce similar quinonoid dihydro species. The quinonoid species derived from 5,6,7,8-tetrahydropteridin-4(3*H*)-one and its 6-methyl and *cis*-6,7-dimethyl derivatives are shown to be viable substrates for human brain dihydropteridine reductase, and apparent K_m and $V_{max.}$ values are reported.

INTRODUCTION

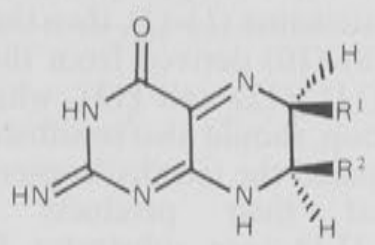
Dihydropteridine reductase (EC 1.6.99.7) catalyses the reduction of quinonoid 7,8-dihydro-L-*erythro*-(6*H*)biopterin (1) to 5,6,7,8-tetrahydro-L-*erythro*-(3*H*)biopterin (4) in the presence of NADH. Simple alkylpterins, e.g. compounds (2) and (3), are effective substrates and can replace the natural compound (1) in this enzymic reaction (Armarego *et al.*, 1984). In Nature quinonoid dihydrobiopterin is produced from the tetrahydro derivative (4) during the hydroxylation of phenylalanine, tyrosine and tryptophan by the enzymes phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4) in the presence of of O_2 to form tyrosine, 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan respectively (Kaufman & Fisher, 1974). Quinonoid dihydropterins can also be formed quantitatively from the respective 5,6,7,8-



(1) R^1 = (2′-*R*, 3′-*S*)-CHOHCHOHMe, R^2 = H
(2) R^1 = Me, R^2 = H
(3) R^1 = R^2 = Me



(4) R^1 = (2′-*R*, 3′-*S*)-CHOHCHOHMe, R^2 = H
(5) R^1 = Me, R^2 = H
(6) R^1 = R^2 = Me



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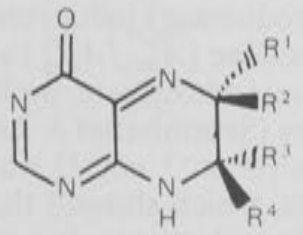
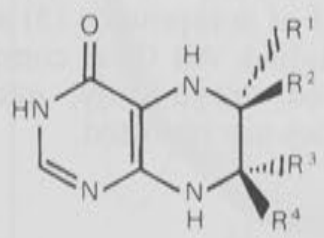


(8) R^1 = R^2 = R^3 = R^4 = H
(9) R^1 = Me, R^2 = R^3 = R^4 = H
(10) R^1 = R^3 = Me, R^2 = R^4 = H



(11) R^1 = R^2 = R^3 = R^4 = H
(11a) R^1 = R^2 = R^3 = R^4 = ^{2}H
(12) R^1 = Me, R^2 = R^3 = R^4 = H
(12a) R^1 = Me, R^2 = R^4 = ^{2}H, R^3 = H
(13) R^1 = R^3 = Me, R^2 = R^4 = H
(13a) R^1 = R^3 = Me, R^2 = R^4 = ^{2}H

Abbreviation used: pterin is the generic name for 2-aminopteridin-4(3*H*)-one.

tetrahydropterins at physiological pH by oxidation with molecular O_2 (Kaufman, 1961; Viscontini & Bobst, 1965; Mager, 1975; Pearson & Blair, 1975; Armarego & Schou, 1978), Br_2 (Lazarus *et al.*, 1982), Fe^{3+} (Archer *et al.*, 1972), $K_3Fe(CN)_6$, 2,6-dichlorophenolindophenol (Kaufman, 1961; Nielsen *et al.*, 1969; Archer *et al.*, 1972), ferricytochrome *c* (Hasegawa *et al.*, 1978) and peroxidase in the presence of H_2O_2 (Nielsen *et al.*, 1969) or O_2 (Armarego *et al.*, 1983*a*). The most commonly used method for generating quinonoid dihydropterins rapidly and quantitatively for assaying dihydropteridine reductase is the Nielsen *et al.* (1969) peroxidase/H_2O_2 method (Craine *et al.*, 1972; Chauvin *et al.*, 1979; Firgaira *et al.*, 1979; Armarego *et al.*, 1983*a*,*b*, 1984; Shen, 1984). The structures of the quinonoid dihydropterin produced by the different oxidants and the aromatic amino acid hydroxylases are the same, and have been the subject of several studies. Kaufman (1964) proposed the *para*-quinonoid structure (7) after a suggestion by Hemmerich (1964), and presented good evidence for the pyrazine moiety of structure (7). This has subsequently been confirmed by several other methods (cf. Armarego *et al.*, 1984). However, the exact tautomeric structure of the pyrimidine ring has been questioned and kinetic evidence presented to show that the tautomer with the endocyclic double bond [i.e. between C-2 and N-3, as in structures (1), (2) and (3)] was the reactive tautomer (Armarego & Waring, 1982, 1983*b*). A study of the chemical shifts of the ^{15}N nuclear magnetic resonances upon oxidizing the tetrahydropterin (6) to the quinonoid dihydropterin labelled with this isotope revealed that in aqueous solution at physiological pH the predominant tautomer is the one with the endocyclic double bond in the pyrimidine ring (3) (Benkovic *et al.*, 1985). We argued that if the quinonoid dihydropterin tautomer that reacts as a substrate for dihydropteridine reductase has the tautomeric structure (1)–(3), then the quinonoid dihydropteridines (8)–(10) derived from the 5,6,7,8-tetrahydropteridines (11), (12) and (13), which do not have the 2-amino group, should also be substrates for this enzyme. We have studied the tetrahydropteridines (11)–(13), and report that their products of oxidation by peroxidase/H_2O_2 are substrates for dihydropteridine reductase and show Michaelis–Menten kinetics from which satisfactory K_m and $k_{cat.}$ values were determined. Further, in order to demonstrate that the quinonoid dihydropteridines (8)–(10) may have tautomeric structures similar to the quinonoid dihydropterins (2) and (3), the oxidation of their respective 5,6,7,8-tetrahydryro derivatives (11), (12) and (13), and of compounds (5) and (6), by peroxidase/H_2O_2 was studied. All these compounds were shown to be oxidized enzymically, and their apparent K_m and $V_{max.}$ values are reported.

MATERIALS AND METHODS

General methods

Kinetic measurements were performed on a Unicam SP.1800 double-beam spectrometer and Rikadenki model B-281H recorder with 5 mV across the slide wire to produce a maximum pen movement corresponding to 0.1 absorbance unit. For higher sensitivity a Cary 219 instrument adjusted to produce maximum pen movement of 0.01 absorbance unit was used, and accurate measurements of absorbances for concentration determinations were performed on a Perkin–Elmer Lambda 1 single-beam spectrometer. T.l.c. was run on silica gel 60 F_{254} (Merck) and eluted with butan-1-ol/acetic acid/water (20:3:7, by vol.). The spots were revealed by u.v. light (at 250 and 365 nm) and staining with I_2 vapour.

Tetrahydropteridines

The tetrahydropteridines (11)–(13a) used were analytically pure and their syntheses and spectroscopic properties are reported elsewhere (Armarego *et al.*, 1986).

Dihydropteridine reductase

The enzyme from human brain was purified as described previously (Armarego & Waring, 1983*a*) but with a freshly prepared naphthoquinone column (Cotton & Jennings, 1978), and gave one band (M_r 26000) on SDS/polyacrylamide-gel electrophoresis without passage through a 5′-AMP–Sepharose column. Protein concentrations were measured with the Bio-Rad Laboratories (Richmond, CA, U.S.A.) standard assay kit and the microassay kit (for concentrations less than 25 μg/ml), with bovine serum albumin (Bio-Rad Laboratories) as standard. This stock enzyme solution (0.39 mg/ml) was divided into 2 ml portions and frozen in glass vials. These can be thawed and refrozen several times without loss of activity, and the frozen solutions can be stored at below -10 °C for more than a year without loss of activity. Dilute solutions (1:10) of this stock (but not stock solutions) lost enzyme activity rapidly when stored in polypropylene vials (but not glass vials), possibly owing to adsorption on the plastic (Randles & Armarego, 1985). Its activity was 81 nmol of NADH oxidized/min per 0.39 μg in an assay mixture containing Tris/HCl buffer (100 μl, 1 M), 6-methyl-5,6,7,8-tetrahydropterin (85 μM), horseradish peroxidase (Boehringer grade II; 10 μg), H_2O_2 (11 mM) and NADH (73 μM) in a total volume of 1 ml at pH 7.4 (25 °C) in the two cuvettes of a double-beam u.v. spectrometer, and the reaction was started by addition of 1 μl of enzyme. The initial rate was measured by the change in absorbance at 340 nm by using ϵ_{340} 6.22×10^3 $M^{-1} \cdot cm^{-1}$ for NADH.

Horseradish peroxidase

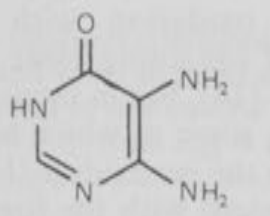
Boehringer grade II horseradish peroxidase (A_{403}/A_{279} ratio 1.0) or Sigma type I horseradish peroxidase (RZ 0.6, used at double the concentration) was used for the generation of quinonoid pterin species in the dihydropteridine reductase kinetic runs. Boehringer grade I horseradish peroxidase [A_{402}/A_{272} ratio 1.35, ϵ_{403} 63×10^3 $M^{-1} \cdot cm^{-1}$ in 1 mM-phosphate buffer, pH 6.6, at 25 °C; literature values (Schonbaum & Lo, 1972) A_{403}/A_{275} ratio 3.25, ϵ_{403} 102×10^3 $M^{-1} \cdot cm^{-1}$] was used for the kinetic measurements, which showed that the tetrahydropteridines were viable substrates for peroxidase I (peroxidase–H_2O_2 complex).

Kinetic measurements

Solutions for the kinetic runs for obtaining the K_m and $V_{max.}$ values with peroxidase at 25 °C contained the following: Tris/HCl buffer, pH 7.4 (100 μl, 1 M), [or potassium phosphate buffer, pH 7.1 (100 μl, 3 M)], substrate (100 μl, concn. 0.5–2 K_m), H_2O_2 (100 μl, 11 or 22 nmol; stock solution concn. determined from $\epsilon_{237} = 67$ $M^{-1} \cdot cm^{-1}$ in water) and water to make 990 μl

(or 1 ml in the blank cuvette). The reaction was initiated by injection of peroxidase (10 μl, 2 μg of Boehringer grade I), and initial rates were obtained by measuring the rate of decrease in absorbance at an analytical wavelength set for each substrate (see Table 2). The non-enzymic rates in 0.1 M-Tris/HCl buffer, pH 7.4, were negligible for compounds (11), (12), (13) and (14) in the absence of H_2O_2, and 8.9, 7.0, 7.6 and 5.9% were oxidized respectively in the first 10 min in the presence of 11 mM-H_2O_2 alone. The amounts oxidized in the first 10 min were slightly less than these when H_2O_2 was 11 μM.

Solutions for the kinetic runs for dihydropteridine reductase activity at 25 °C contained the following: Tris/HCl buffer, pH 7.4 (100 μl, 1 M) [or potassium phosphate buffer, pH 7.1 (100 μl, 3 M)], peroxidase (100 μl, 20 μg of Boehringer grade II or 40 μg of Sigma type I enzyme), H_2O_2 (100 μl, 11 μmol), enzyme (1–3 μl, 0.39–1.17 μg, of stock or 10 times these values of 1:10-diluted enzyme, added to one cuvette), NADH (30 μl, 2.7–4.4 mM; Sigma Chemical Co.; see Table 3), substrate (30 μl, concn. 0.5–3 K_m) and water to make 1 ml. The first three components were made as a stock solution (kept at 0–2 °C during runs), enzyme was injected into one cuvette (with a Hamilton Whittier-cal PB600 dispenser with a 50 μl Hamilton syringe delivering 1 μl at a time), followed by simultaneous addition of NADH to both cuvettes (by placing the solutions in two small platinum baskets attached to a metal beam and moving them up and down in the solution eight times). After mixing, the recorder was activated to ensure that the absorbance remained unaltered for approx. 1 min. The reaction was initiated by simultaneous addition of substrate (as in the case of NADH addition above), and the initial rates were obtained from the rate of change of absorbance at 340 nm for NADH oxidation. Rates from duplicate runs from at least five concentrations of substrates (see above) were used to derive the kinetic parameters. All these parameters were calculated with the aid of a computer program kindly supplied by Dr. A. Cornish-Bowden (1981), and values derived from double-reciprocal plots of concentrations versus initial velocities agreed favourably with computed values. The pH values at the end of each run in this section were measured with a glass electrode and were identical with the values of the stock buffers originally used.



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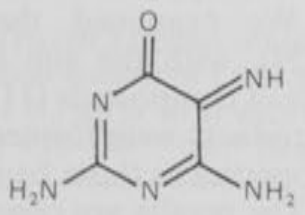

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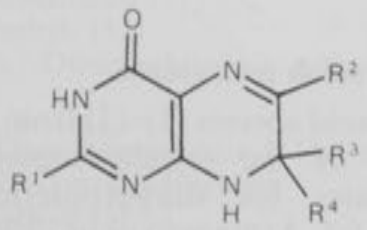

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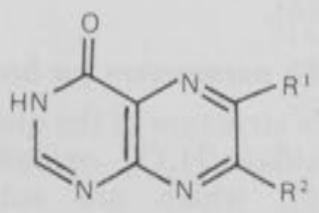


(17) R^1 = Me, R^2 = H

(18) R^1 = R^2 = Me

RESULTS AND DISCUSSION

Nature of the substrates for dihydropteridine reductase

The 5,6,7,8-tetrahydropteridin-4(3*H*)-ones [(11)–(13), pK_a approx. 3.8; cf. Albert & Matsuura (1962)] do not undergo aerobic oxidation in aqueous buffer at approx. pH 7 as readily as the more strongly basic 5,6,7,8-tetrahydropterins [e.g. (5) and (6), pK_a approx. 5.6; cf. Bobst & Viscontini (1966), Kaleen & Jencks (1968), Matsuura & Sugimoto (1970) and Pfleiderer & Mengel (1971)] even though they exist entirely as neutral species. In this solution the pteridines (11)–(13) show virtually no changes in u.v. spectra in 1 h. Addition of 50–70 mol-equivalents of H_2O_2 causes changes in spectra, but they are too slow (less than 5% change in 10 min) for deriving kinetic parameters. However, when peroxidase and H_2O_2 are added together very rapid spectral changes occur (Fig. 1). The spectral changes for each compound are slightly different but all showed the same pattern, i.e. a fall in intensity of the major peak such that finally after 24 h the main u.v.-absorbing band had disappeared leaving a very weak band at long wavelength (approx. 380 nm). These are very similar to those observed with 4,5-diaminopyrimidin-6(1*H*)-one (14) and 2,4,5-triaminopyrimidin-6(1*H*)-one under the same conditions. The latter is known to give the quinonoid pyrimidine (15), which is a good substrate for dihydroptridine reductase (Armarego *et al.*, 1983*b*). These changes were found to obey first-order kinetics for more than 85% of the reaction, and the rate constants and half-lives are listed in Table 1.

Preliminary assays of the peroxidase/H_2O_2 oxidation products of compounds (11)–(14), without simultaneous addition of reagents to the reaction and reference cuvettes, indicated that the oxidation products were substrates for dihydropteridine reductase, but the reproducibility of the rate traces was very poor. We were

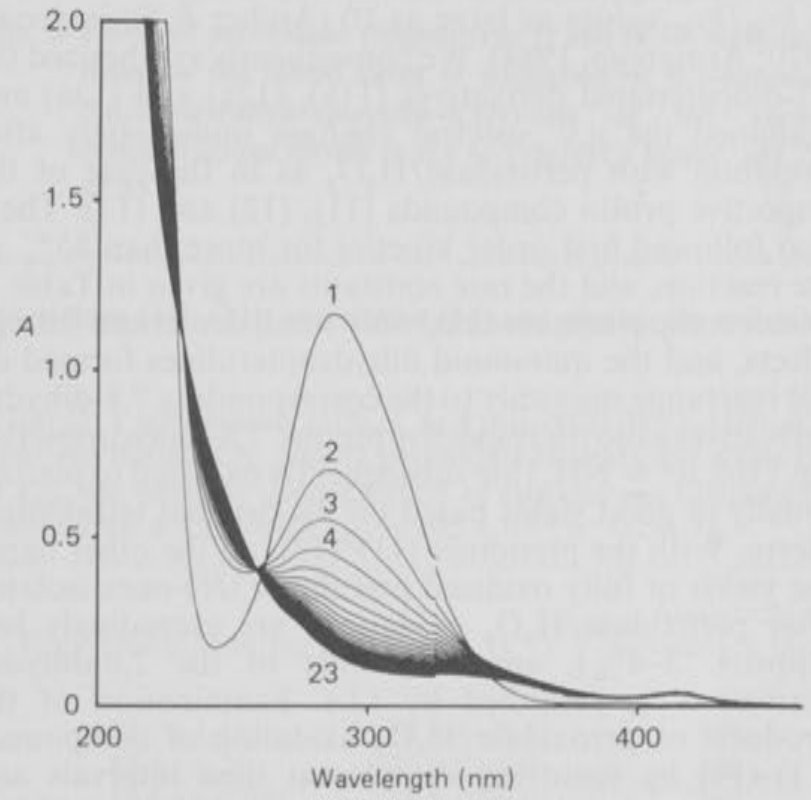


Fig. 1. U.v. spectra of 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (117 μM) after treatment with peroxidase (20 μg) and H_2O_2 (11 μmol) in 0.1 M-Tris/HCl buffer, pH 7.4, at 25 °C

Scans 1–4 and 23 are after 0, 0.66, 2.3, 3.8 and 35 min respectively.

Table 1. First-order rate constants for the oxidation of compounds (11)–(14) with horseradish peroxidase/H_2O_2 at 25 °C

Reaction mixtures contained Tris/HCl (unless otherwise indicated (100 μl; 1 M), substrate (100 μl), H_2O_2 (100 μl, 11 μmol) and water (600 μl), and the reaction was initiated with peroxidase (100 μl; 20 μg) at pH 7.3. Measurements were made with a single-beam spectrometer.

Compound	Final concn. (μM)	Analytical wavelength (nm)	$10^{-3} \times k_{obs.}$ (s^{-1})	$t_{\frac{1}{2}}$ (min)
(11)	149	289	4.95 (±0.08)	2.3
(11a)	187	289	6.19 (±0.07)	1.9
(12)	143	290	2.07 (±0.02)	5.6
(12)*	117	290	1.92 (±0.02)	6.0
(12)†	117	290	0.82 (±0.01)	13.9
(12a)	173	292	2.10 (±0.02)	5.5
(13)	172	292	2.12 (±0.04)	5.5
(13)*	166	292	1.20 (±0.01)	9.6
(13)†	166	292	0.39 (±0.01)	29.4
(13a)	256	292	2.32 (±0.04)	5.0
(14)	190	282	6.07 (±0.01)	1.9
(14)‡	190	282	8.22 (±0.01)	1.4
(14)†	190	282	7.11 (±0.08)	1.6

* In 0.1 M-Bicine buffer, pH 7.0.
† In 0.3 M-potassium phosphate buffer, pH 7.0.
‡ In 0.1 M-Bicine buffer, pH 6.7.

concerned that this might be due to the rearrangement of the rapidly formed quinonoid species (8)–(10) to the assumed thermodynamically more stable 7,8-dihydropteridines (16, $R^1 = H$). In the pterin series this rearrangement occurs readily, e.g. (3)→(16, $R^1 = NH_2$, $R^2 = R^3 = Me$, $R^4 = H$), and is subject to general acid/base catalysis. It was also shown that when H-6 in alkylpterins [e.g. compounds (2) and (3)] was replaced by deuterium then the rate of rearrangement of quinonoid species to the 7,8-dihydro derivatives [e.g. (16), $R^1 = NH_2$] in Tris/HCl buffer at approx. pH 7 is subject to k_{1H}/k_{2H} values as large as 10 (Archer & Scrimgeour, 1970; Armarego, 1984). We consequently synthesized the 6,7-dideuteriated derivatives (11a), (12a) and (13a) and examined the u.v. spectral changes immediately after oxidation with peroxidase/H_2O_2 as in the case of the respective protio compounds (11), (12) and (13). These also followed first-order kinetics for more than 85% of the reaction, and the rate constants are given in Table 1. Direct comparison revealed only small deuterium isotope effects, and the quinonoid dihydropteridines formed do not rearrange smoothly to the corresponding 7,8-dihydro derivatives as do the respective pterins. 7,8-Dihydropterins [i.e. (16), $R^1 = NH_2$] are subsequently oxidized to pterins, usually in good yields based on the original tetrahydropterin. With the pteridines (11)–(13), on the other hand, the yields of fully oxidized pteridin-4(3*H*)-ones isolated after peroxidase/H_2O_2 oxidations are exceedingly low (approx. 3–4%), and very little of the 7,8-dihydro derivatives is identified by t.l.c. Examination of the products of peroxidase/H_2O_2 oxidation of compounds (11)–(14) by removing samples at time intervals and separating the components by t.l.c. showed that within a minute of mixing at least seven spots appeared, and that their relative intensities varied with time but the number of spots did not decrease with time. The reaction was repeated on a larger scale for compounds (12) and (13), allowed to proceed for 24 h and the products were separated by t.l.c. One band in each case was identified conclusively by u.v. spectra at three pH values, by ^{1}H-n.m.r. spectra and by t.l.c. (re-run) as the fully oxidized compounds (17) and (18). Most of the other bands gave substances, in small amounts, that had no u.v. absorption and a few unidentified proton signals in the n.m.r. spectra. Similar oxidations were performed in the n.m.r. tube and the proton spectra were examined at time intervals. After 3–5 min the downfield signal from H-2 disappeared whereas the upfield signals from the methyl groups persisted longer. These results indicate that oxidation at C-2 could have occurred to form, say, lumazines, and/or the pyrimidine ring was disrupted by hydration across the C-2=N-1 double bond followed by ring opening. The lack of u.v. absorption of some of the bands is indication of ring cleavage. In the case of the pyrimidine (14) similar results were obtained (i.e. several spots on t.l.c.), but we could not identify 2-carboxy-1,3,5-triazin-4(3*H*)-one or 1,3,5-triazin-2,4(1*H*,3*H*)-dione among the products. These would have been the possible products if the oxidation proceeded as that of 2,4,5-triaminopyrimidin-6(1*H*)-one (Jongejan *et al.*, 1983). We examined the oxidation with Br_2 and $K_3Fe(CN)_6$ with the aim of obtaining clear oxidation products of compounds (11)–(14), but in these cases also many products were formed, some of which had similar t.l.c. properties as those from the peroxidase/H_2O_2 oxidations. The results are consistent with the formation of a primary oxidation product (such as quinonoid dihydropteridine) that subsequently undergoes degradation to products that may react further with the oxidant.

Kinetic parameters for horseradish peroxidase

The structure of the quinonoid species (1)–(3) from the peroxidase/H_2O_2 oxidation of the tetrahydropterins (4)–(6), which are substrates for dihydropteridine reductase, is well established (cf. Armarego *et al.*, 1984). Although these species rearrange to the corresponding 7,8-dihydro(3*H*)pterins (16, $R^1 = NH_2$), they are stable

enough for some structural investigations. They can be generated so rapidly (e.g. within 20 s in assay conditions; see the Materials and methods section) that intermediates cannot be detected under the present assay procedures. The pteridin-4(3*H*)-ones (11)–(13) are also oxidized with peroxidase alone (or in buffer saturated with O_2), but very slowly (less than 0.1% in 10 min) compared with the oxidation of the pterins (5) and (6). The main difference in behaviour between the pterins and the pteridin-4(3*H*)-ones is that the quinonoid species from the latter are less stable and distinct species cannot be observed.

The close structural similarities of the tetrahydropterins (5) and (6) and the tetrahydropteridin-4(3*H*)-ones (12) and (13) prompted the suggestion that their oxidation products (2) and (3) and (9) and (10) should have the same structures and that differences in behaviour are due to the differences in oxidation potential between the two series (i.e. with and without the 2-amino group). In order to obtain further evidence to support this suggestion we tested both series of compounds to see if they were substrates for peroxidase/H_2O_2 and exhibited Michaelis–Menten kinetics. We found that this was the case. The u.v. spectrum of the peroxidase used was similar to that of the reduced enzyme, whereas in the presence of more than 1 equivalent of H_2O_2 it was converted into peroxidase I (cf. Schonbaum & Lo, 1972), a process that is known to be very fast (second-order rate constant 0.9×10^7 M^{-1}·s^{-1}; Chance, 1949; Kato *et al.*, 1984). A preliminary study with a constant amount of peroxidase and H_2O_2 with various amounts of compounds (5), (6), (11), (12), (13) and (14) revealed that the rates of oxidation of all these compounds exhibited saturation kinetics, i.e. the rates increased with increasing concentration of substrate until they reached a maximum value. A detailed investigation was undertaken of the rates of oxidation of all these substrates with various amounts of peroxidase and of H_2O_2, and keeping the concentration of one of these two components constant while altering that of the other. A typical series of curves is shown in Fig. 2. The rates increase linearly up to a concentration of peroxidase of 8 μg/ml. The rates increased as the H_2O_2 concentration increased up to approx. 100 μM, and then the rates decreased, most probably owing to deterioration of the enzyme (Schonbaum & Lo, 1972). For the measurements of the kinetic parameters we settled on a low concentration of H_2O_2 (11 μM). From the plots of this concentration of H_2O_2 we chose enzyme concentrations from the linear portions of the curve where the slope was such that doubling the amount of enzyme doubled the rate of reaction. The kinetic parameters for the enzyme with the above substrates are listed in Table 2, together with the concentrations of the reagents used.

The K_m and $k_{cat.}$ values obtained show that the enzymic oxidation of all these substrates obeys Michaelis–Menten kinetics. The orders of these parameters imply

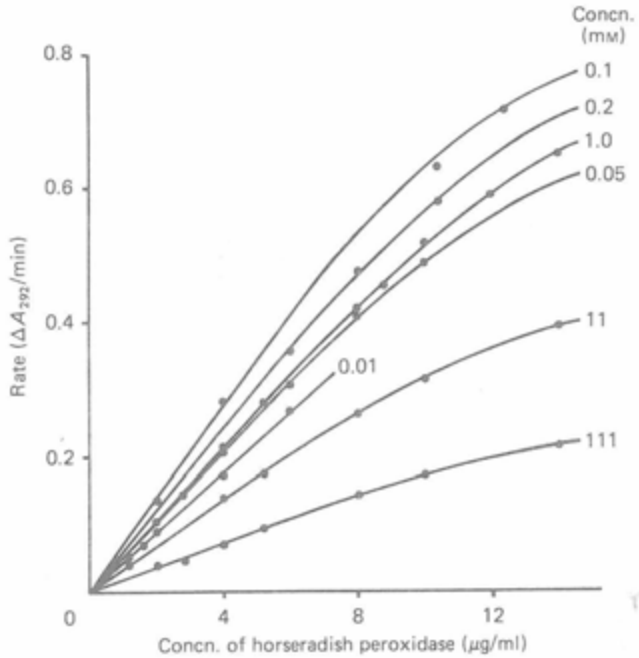


Fig. 2. Effect of peroxidase (Sigma type I) and H_2O_2 concentrations on the initial rates of oxidation of 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one at the various concentrations shown in 0.1 M-Tris/HCl buffer, pH 7.4, at 25 °C

Table 2. Kinetic parameters of horseradish peroxidase/H_2O_2 with the pteridines (11)–(13), pyrimidine (14) and pterins (5) and (6) at pH 7.4 and 25 °C

Reaction mixtures contained Tris/HCl (unless otherwise indicated) (100 μl; 1 M, substrate (100 μl, in 4 mM-HCl), H_2O_2 (100 μl; 110 μM) and water to make a final volume of 1.000 ml, and the reaction was initiated by adding peroxidase (Boehringer grade I) (2 μg in 10 μl to one cuvette or 0.2 μg in 10 μl with the pterins). For $k_{cat.}$ the effective concentration of enzyme was calculated to be as 61.8% from $\epsilon_{403} = 63 \times 10^{-3}$ M^{-1}·cm^{-1} [literature value (Schonbaum & Lo, 1972) $\epsilon_{403} = 102 \times 10^{-3}$ M^{-1}·cm^{-1}].

5,6,7,8-Tetrahydropteridin-4(3*H*)-one	K_m (μM)	$V_{max.}$ (μmol/min per mg)	$k_{cat.}$ (s^{-1})	Analytical wavelength (nm)
Unsubstituted (11)	173 (±3)	143 (±1)	58.6 (±0.5)	289
6-Methyl- (12)	314 (±20)	53.4 (±2)	22.0 (±0.9)	290
cis-6,7-Dimethyl- (13)	44.9 (±0.2)	16.1 (±0.03)	6.6 (±0.01)	292
	28.6 (±0.4)*	11.6 (±0.09)*	4.8 (±0.04)*	292
4,5-Diaminopyrimidin-6(1*H*)-one (14)	203 (±8)	149 (±3)	55.6 (±1.4)	282
5,6,7,8-Tetrahydropterin				
6-Methyl- (5)	45.7 (±1.1)	27.9 (±0.4)	11.5 (±0.1)	298
cis-6,7-Dimethyl- (6)	103 (±2)	50.9 (±0.8)	21.0 (±0.3)	298

* In 0.3 M-potassium phosphate buffer, pH 7.1.

that the mechanisms of enzymic oxidation of the tetrahydropteridin-4(3*H*)-ones (11), (12) and (13) are probably similar to those of the tetrahydropterins (5) and (6), and that the initial products formed are the quinonoid species (8)–(10), (2) and (3) respectively.

Kinetic parameters for dihydropteridine reductase

In the first enzymic experiments we ventured to see if the peroxidase/H_2O_2 oxidation products of compounds (11)–(13) were inhibitors of dihydropteridine reductase. We found no inhibition at concentrations of 120–140 μM of the products when quinonoid 6-methyl-7,8-dihydro-(6*H*)pterin (2) (at 49 μM) and NADH (at 58 μM) were used. Incubation of compounds (11)–(13) in the presence of peroxidase/H_2O_2 for various times up to 6 half-lives (see Table 1) also showed no decrease in the initial rates with quinonoid pterin (2) and NADH. Preliminary studies, however, showed that one of the products of the peroxidase/H_2O_2 oxidation products of each of compounds (11)–(14) was a substrate for dihydropteridine reductase.

The kinetic properties of these substrates (shown in Table 1) made it difficult to use standard mixing procedures because the decomposition of the initial quinonoid species formed can proceed to quite an extent in one cuvette before addition of an ingredient in the other cuvette. It was imperative that addition of both the substrates (11)–(14) and the NADH solutions were respectively added simultaneously to both cuvettes. After experimenting also with the order of addition of reagents we selected the order that gave best reproducibility of initial rate traces for a particular series of runs. Because the initial products of peroxidase/H_2O_2 oxidation of the substrates (11)–(14) undergo decomposition readily, it was necessary to find out how the dihydropteridine reductase activity altered with time after allowing the decomposition to proceed before starting the assay for this enzyme. In this case the order of addition was stock solution (Tris/HCl, peroxidase, H_2O_2), dihydropteridine reductase (in one cuvette only), substrate (11)–(14) (simultaneously to both cuvettes) and allowing a recorded time interval before initiation of the reductase reaction by adding NADH (simultaneously to both cuvettes) and recording the spectral change at 340 nm. The curve for 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one is shown in Fig. 3. Maximum activity was also observed at the shortest time with compounds (11), (13) and (14), and all subsequent assays were performed at the shortest possible time (approx. 1 min). The same procedure was used for the measurement of the kinetic parameters except that the order of simultaneous addition of compounds (11)–(14) and NADH was reversed, i.e. the reactions were initiated with compounds (11)–(14). In this order, the oxidation of the tetrahydropteridines by peroxidase/H_2O_2 may appear to be inhibited by large concentrations of NADH. This is because the quinonoid dihydropteridine is reduced back

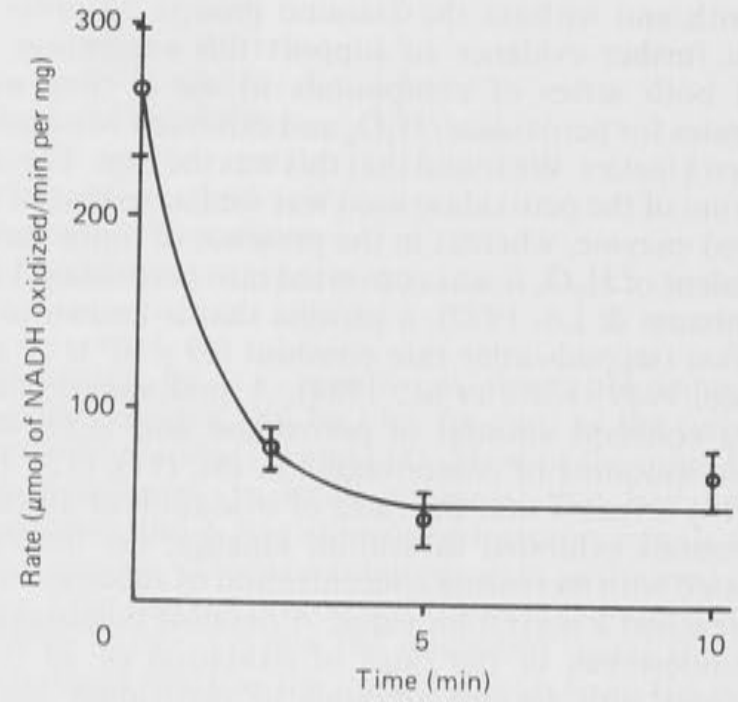


Fig. 3. Dihydropteridine reductase (59 ng) activity at various time intervals after initiation of the peroxidase/H_2O_2 oxidation of 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (988 μM) and starting the reaction with NADH (79 μM) in 0.1 M-Tris/HCl buffer, pH 7.4 (total volume 1 ml), at 25 °C

Table 3. Kinetic parameters for compounds (8)–(10) with human brain dihydropteridine reductase at 25 °C

Solutions were mixed in the order buffer (100 μl; 10 times the concentration given below), peroxidase (100 μl; 40 μg), H_2O_2 (100 μl; 11 μmol) and water to make 1.000 ml after all ingredients are added (these four are used as a stock solution), dihydropteridine reductase (7 μl of 1/10-diluted enzyme, to 3 μl of stock enzyme injected, to one cuvette only), NADH (30 μl, simultaneously to the two cuvettes) and allowed to equilibrate. Reaction is initiated by addition of tetrahydropteridines (11)–(13) (30 μl) simultaneously to both cuvettes.

Substrate	Apparent K_m (μM)	Apparent $V_{max.}$ (μmol/min per mg)	Concn. of NADH (μM)	Concn. of enzyme (μg/ml)
0.1 M-Tris/HCl buffer, pH 7.4				
(8) from (11)	669 (±2)	234 (±0.4)	117	0.39
(9) from (12)	366 (±20)	131 (±3)	117	0.39
(10) from (13)	754 (±45)	76 (±4)	117	1.17*
0.3 M-Potassium phosphate buffer, pH 7.1				
(8) from (11)	597 (±20)	318 (±8)	117	0.39
(9) from (12)	276 (±8)	444 (±7)	80	0.27
(10) from (13)	640 (±52)	489 (±27)	131	0.31

* Close to, but not on, the linear part of the plot of rate versus enzyme concentration.

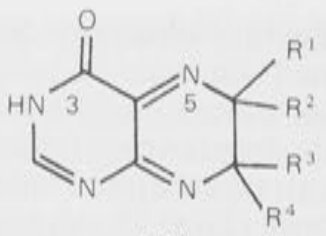
to the starting tetrahydropteridines by NADH, the reduction being faster the higher the concentration of NADH and pteridine. We have studied the rates of non-enzymic reduction of the quinonoid species (2), (3) and (8)–(10) with the use of a single-beam spectrometer and found that at pH 7.4 the rates were 1.4–6.8% of the enzymic rates. The rates of formation of quinonoid species are not rate-limiting. Note also that the non-enzymic rates are compensated in the double-beam spectrometer and are neglected, and hence only the enzymic rates are observed. Below pH 6.8, however, the non-enzymic rates increase sharply because the quinonoid species become protonated (pK_a approx. 5.4 for the pterins; see below). These compounds were clearly oxidized to transient species that exhibited Michaelis–Menten kinetics with dihydropteridine reductase and the appropriate apparent K_m and $V_{max.}$ values that we measured are in Table 3.

For meaningful kinetic parameters the concentrations of dihydropteridine reductase were selected from plots of enzyme activity versus enzyme concentration. In all cases the plots were linear up to enzyme concentrations of 0.4–0.6 mg/ml, i.e. the rate was doubled when the enzyme concentration was doubled, and then slowly levelled off. This levelling off of enzyme activity does not appear to be caused by inefficient recycling of the pteridines by peroxidase/H_2O_2 and may be due to lower effective concentration of enzyme caused by association or polymerization, but we have not investigated this further. The enzyme concentrations used were selected from the linear portions of the plots. Although we found that the quinonoid species of the 4,5-diaminopyrimidinone (14) had substrate activity, these decomposed too rapidly (see Table 1), and the reproducibility of the initial-rate plots was very poor.

The parameters were measured in Tris and phosphate buffers because it was found that the rates of oxidation were lower in the latter buffer. We do not know precisely what is happening in this reaction, but we know that the quinonoid species are formed rapidly and then decompose. The plot in Fig. 3 confirms this because the dihydropteridine reductase activity of the species formed from peroxidase/H_2O_2 oxidation is highest immediately (approx. 1 min) after mixing and then decreases. The rate constants in Table 1 are therefore for the formation and degradation of the quinonoid species. Since we do not know the individual rate constants of formation and degradation of the active species, we cannot take the concentrations of the respective quinonoid species in Table 3 as being the same as those of the tetrahydropteridines (11), (12) and (13) from which they were derived. Therefore the apparent K_m values are maximum values for these substrates and the true values could be much smaller. The reproducibility of the initial rates and the satisfactory spread of errors confirms that the proportional concentrations of quinonoid species with respect to tetrahydropteridines used, at various concentrations of the latter, are within experimental error in each set of experiments.

The K_m values in phosphate buffer are slightly less than in Tris buffer and could reflect the higher proportion of quinonoid species (slower decomposition) in the former buffer. This does not affect the apparent $V_{max.}$ values, which are higher in phosphate buffer. The concentrations of NADH in Table 3 are at or close to saturating values because when they are doubled no significant increases in rates are observed. Some of the substrates contained small amounts of borate salts, and we were concerned that borate might inhibit the enzyme. No inhibition of dihydropteridine reductase was observed at borate concentrations up to 1 mM when 6-methyl-7,8-dihydro(6*H*)-pterin (2) (at 55 μM) and NADH (100 μM) were used. The highest concentration of borate from the substrates in the assays was 0.9 mM, and most of the solutions contained considerably less than this concentration.

In conclusion, we have shown that the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones (11), (12) and (13) are effective substrates for horseradish peroxidase in the presence of H_2O_2, similarly to the related 2-amino derivatives, the 5,6,7,8-tetrahydropterins (5) and (6). The transient quinonoid species derived from the oxidation of the tetrahydropteridin-4(3*H*)-ones (11)–(13) are found to be substrates for dihydropteridine reductase. A 2-amino group is not essential for substrate activity with the latter enzyme but most probably enhances the binding. The data show that the quinonoid structures (2) and (3), but not (7), are probably the reactive species in the dihydropteridine reductase-catalysed oxidation of NADH. The proposition that the quinonoid structures derived from compounds (11)–(14) are different from those derived from the pterins (2) and (3) when the same oxidant (peroxidase/H_2O_2) is used, and are also viable substrates for a second enzyme (dihydropteridine reductase), is very unlikely. Such compounds, however, could have the *ortho*-quinonoid structure (19), a structure with energy comparable with that of the tautomers (2) and (3) (Gready, 1985), but this has been ruled out by other studies (Armarego & Waring, 1982; Lazarus *et al.*, 1982; Benkovic *et al.*, 1985). The u.v.-spectral changes of 2,5-diaminopyrimidin-4(3*H*)-one (20) on treatment with peroxidase/H_2O_2 are very similar to those of the pyrimidinone (14) and the pteridines (11)–(13) (see Fig. 1), and would exclude a quinonoid species with bond arrangements such as (19). Moreover, the pyrimidinone (20) after peroxidase/H_2O_2 treatment exhibited some activity with dihydroperidine reductase. Structure (19) for the pteridin-4(3*H*)-ones implies a remote possibility that dihydropteridine reductase can reduce two different tautomers of very similar substrates. It is true that the cations of the two tautomers are mesomeric, (21) $\rightleftharpoons$ (22), and protonation of the quinonoid species will enhance hydride transfer to N-5 considerably. However, the enzyme will still need to transfer a proton to N-3 in one case (the pterins) and to N-8 in the other case (the pteridinones) after binding of the neutral species, if one postulates different tautomeric structures. If the pteridine binds as the cation, on the other hand, it does not matter which tautomer predominates in neutral solution. The pK_a of the quinonoid dihydropterin (3) is 5.4 (W. L. F. Armarego, unpublished work; cf. also Bailey & Ayling, 1983) and at pH 7.4 it is almost entirely in the unprotonated form. The absence of a 2-amino group from the corresponding quinonoid dihydro(6*H*)pteridin-4-one (10) would be base-weakening and make its pK_a lower than 5.4 by at least 1.6 pH units [compare the pK_a values of 5,6,7,8-tetrahydropteridin-4(3*H*)-ones approx. 3.8; Albert & Matsuura, 1962) and 5,6,7,8-tetrahydropterins (approx. 5.6; Matsuura & Sugimoto, 1970; Pfleiderer & Mengel, 1971)]. These species are even less likely to be protonated at pH approx. 7.4. Protonation of quinonoid species would most probably take place on the enzyme after or before hydride transfer, but this informa-



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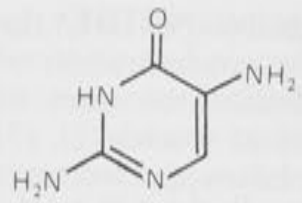

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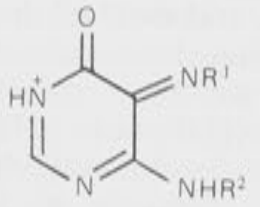

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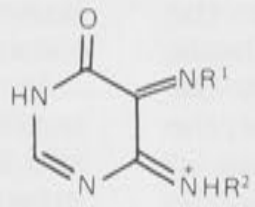

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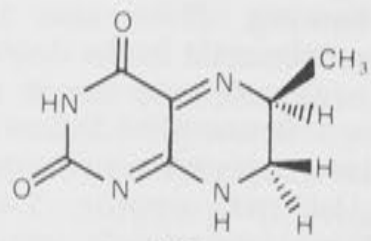

(23)

tion is not known at present. Pulse–chase and stopped-flow kinetic studies of the mechanism of the reactions at the active site of dihydropteridine reductase have revealed that NADH binds first and very tightly, followed by the quinonoid dihydropterin, and that the rate-determining step is a conformational change of the ternary complex, probably assisting in or associated with substrate protonation, immediately before the faster hydride transfer (no deuterium isotope effect) from NADH to the quinonoid dihydropterin, followed by release of products (Poddar & Henkin, 1984). A study of the profiles of the kinetic parameters plotted versus pH could tell us more about the details of the protonation process. Finally, we have examined 6-methyl-7,8-dihydro(6*H*)lumazine (23), which is relatively stable and whose structure has been established by n.m.r. spectroscopy (Lazarus *et al.*, 1982), and found that at 41 μM it is neither a substrate nor an inhibitor of this enzyme.

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Reduced 2-Methyl-4-oxo, 2-Methylamino-4-oxo, 2-Methylthio-4-oxo and 2-Amino-4-thioxo 6,7-Dimethylpteridines, and 6,8-Dimethylpterin as Substrates for Dihydropteridine Reductase

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Abstract

The syntheses of unsubstituted (4), 6-methyl- (6), *cis*-6,7-dimethyl- (8), *cis*-2,6,7-trimethyl- (10), *cis*-6,7-dimethyl-2-thioxo-(1H)- (12) and *cis*-6,7-dimethyl-2-methylthio- (13) 5,6,7,8-tetrahydropteridin-4(3*H*)-one and the deuterated derivatives (5), (7), (9) and (14), and a *cis* and *trans* mixture of 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-thione (11) are described. The existence of the transient quinonoid species (15)–(17) had been previously demonstrated by showing that they are substrates for the enzyme dihydropteridine reductase from human brain. We report that the transient quinonoid species *cis*-2,6,7-trimethyl- (18), *cis*-2-methylthio-6,7-dimethyl- (25), *cis*-2-methylamino-6,7-dimethyl- (26), and 2-amino-6,8-dimethyl- (29) 7,8-dihydropteridin-4(6*H*)-one, and 2-amino-6,7-dimethyl-7,8-dihydropteridin-4(6*H*)-thione (28), but not *cis*-6,7-dimethyl-2-thio-7,8-dihydropteridin-4(6*H*)-one (27) are substrates for this enzyme showing that a variety of substituents in position 2 of the pteridine ring can be tolerated, and confirming that the predominant tautomer (1) of the quinonoid species in aqueous solution is also the reactive tautomer at the active site of this enzyme.

Introduction

Several quinonoid dihydropterins are active substrates for the enzyme dihydropteridine reductase and can exit in three possible tautomeric forms (1), (2) and (3) in aqueous solution at near neutral pH.[1] ^{15}N n.m.r. spectral studies of ^{15}N enriched 6,7-dimethylpterins have shown that species (1) are predominant in solution,[2] and tautomer (3) could not be detected in the ^{1}H n.m.r. spectra.[3] However, the predominant tautomer in solution is not necessarily the structure that is enzymically active. In an endeavour to deduce the tautomeric structure of pterin substrates at the active site we have synthesized the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones (4)–(10), and the related thioxo and methylthio compounds (11)–(14) which we now report together with their spectral properties. The substrate activities of the transient quinonoid species (15)–(17), derived by oxidation of the tetrahydropteridinones (4), (6) and (8),

[1] Armarego, W. L. F., Randles, D., and Waring, P., *Med. Res. Rev.*, 1984, **4**, 267.
[2] Benkovic, S. J., Sammons, D., Armarego, W. L. F., Waring, P., and Inners, R., *J. Am. Chem. Soc.*, 1985, **107**, 3706.
[3] Lazarus, R. A., DeBrosse, C. W., and Benkovic, S. J., *J. Am. Chem. Soc.*, 1982, **104**, 6871.

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towards dihydropteridine reductase have been reported,[4] and the activities of the quinonoid pteridinone (18), the thio compounds (25), (27) and (28), and the pterin (26) are briefly described here. The biological activities strongly support the tautomeric structure (1) as the enzymically active species, and show that the enzyme can tolerate quinonoid dihydropteridines with 2-methyl-4-oxo (18), 2-methylamino-4-oxo (26), 2-methylthio-4-oxo (25), 2-amino-4-thioxo (28) and only 4-oxo (15)–(18) substituents in addition to the 2-amino-4-oxo substituents that are present in the natural substrate, quinonoid 7,8-dihydro(6*H*)biopterin and analogues such as (1).[1]

We have indicated in the Experimental section where our syntheses differed from published procedures and have included unpublished spectral data. Only new preparations and unusual features of the syntheses will be discussed here.

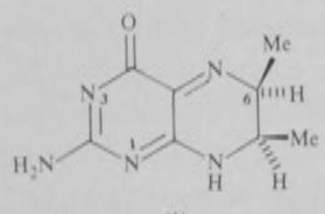
Preparation of Pteridin-4(3*H*)-ones

Pteridin-4(3*H*)-one could not be reduced catalytically with pre-reduced platinum oxide in trifluoroacetic acid or methanol, or with palladium on charcoal in ethanol. This was not due to poisoning of the catalyst by traces of sulfur from the intermediates because the reductions were unsuccessful with rigorously purified pteridinone, and when large excesses of catalyst were used. We have no satisfactory explanation for this failure. Reduction to the tetrahydro derivative (4) with sodium borohydride, however, was successful.[5]

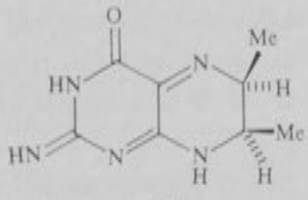
6-Methylpteridin-4(3*H*)-one, on the other hand, gave the 5,6,7,8-tetrahydro derivative (6) on hydrogenation with platinum oxide in methanol. When the reduction was carried out in trifluoroacetic acid and stopped after two equivalents of hydrogen were absorbed, a mixture of tetra- and hexahydro-pteridinone was formed. The pure hexahydro derivative (19) was obtained by allowing this reduction to proceed to completion. Alternative structures for the hexahydro product, such as the ring-opened cations of the *N*-methyltetrahydropyrazines (20) or (21), or of the *N*-hydroxymethyltetrahydropyrazines (22) or (23) derived from the bicyclic compound (19), were excluded on the grounds that the ^{1}H n.m.r. spectra in acidic solution had proton integrals consistent with two and not three H 2 protons for the singlet at 4·99 ppm [compare with the singlets at 4·30 and 4·33 ppm of the two H 2 protons in the spectra of the neutral species of *cis*- and *trans*-1,2,4a,5,6,7,8,8a-octahydroquinazolin-4(3*H*)-one].[6] Moreover, the ^{13}C n.m.r. chemical shift of C 2 was at 52·1 ppm consistent with a $-HNCH_2NH-$ structure (19) rather than $-HNCH_2OH$ structures such as (22) and (23). This deduction was arrived at from the known ^{13}C chemical shifts of 6-aminohexan-1-ol where C 1 and C 6 are at 62·9 and 43·2 ppm respectively.[7] The model shows that the oxygen adjacent to the methylene carbon atom deshields it much more strongly than an adjacent nitrogen atom. The value of 52·1 ppm observed cannot be consistent with a $-NHCH_2OH$ structure.

cis-6,7-Dimethyl- (8) and *cis*-2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (10), unlike the above, were prepared as their hydrochloride salts by allowing the catalytic reduction of the respective pteridinone in acidic medium to proceed to

[4] Armarego, W. L. F., Ohnishi, A., and Taguchi, H., *Biochem. J.*, 1986, **234**, in press.
[5] Albert, A., and Matsuura, S., *J. Chem. Soc.*, 1962, 2162.
[6] Armarego, W. L. F., and Kobayashi, T., *J. Chem. Soc. C*, 1971, 238.
[7] Formacek, V., Desnoyer, L., Kellerhals, H. P., Keller, T., and Clerc, J. T., *Bruker ^{13}C Data Bank*, 1976, **1**, 139.



(1)



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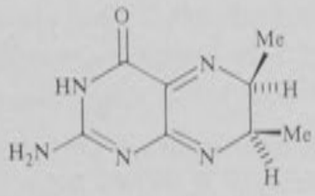

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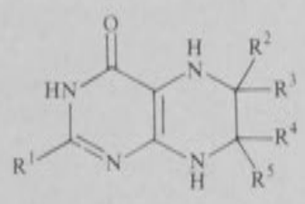
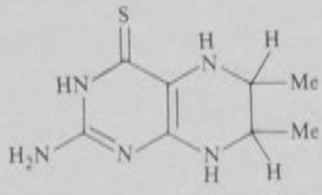
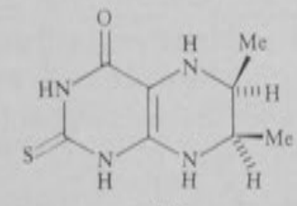


	R^1	R^2	R^3	R^4	R^5
(4)	H	H	H	H	H
(5)	H	D	D	D	D
(6)	H	Me	H	H	H
(7)	H	Me	D	H	D
(8)	H	Me	H	Me	H
(9)	H	Me	D	Me	D
(10)	Me	Me	H	Me	H



(11)



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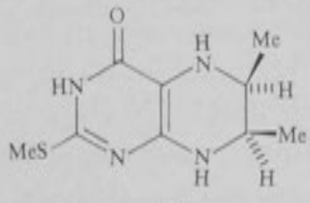
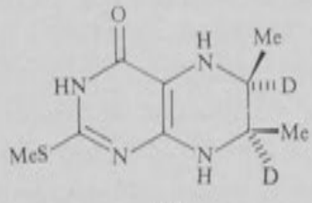
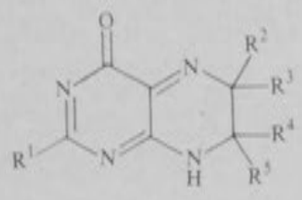


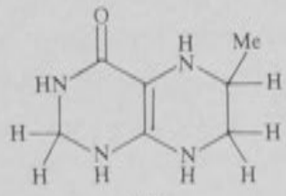
(13)



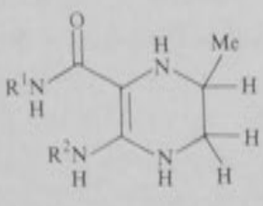
(14)



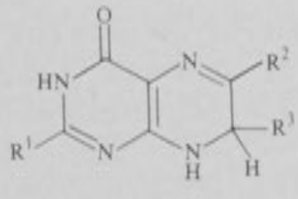
	R^1	R^2	R^3	R^4	R^5
(15)	H	H	H	H	H
(16)	H	Me	H	H	H
(17)	H	Me	H	Me	H
(18)	Me	Me	H	Me	H



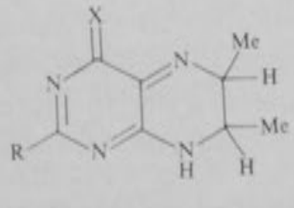
(19)



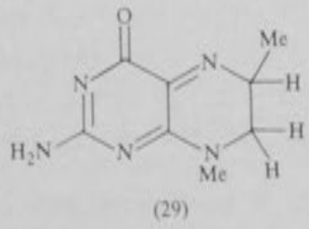
	R^1	R^2
(20)	H	Me
(21)	Me	H
(22)	H	CH_2OH
(23)	CH_2OH	H



(24)



	R	X
(25)	MeS	O
(26)	MeNH	O
(27)	SH	O
(28)	NH_2	S



(29)

completion. The *cis* stereochemistry at C6 and C7 was deduced from the small H6–H7 coupling constants (cf.[8]).

Preparation of Thiopteridines

The successful reduction of pteridin-4(3*H*)-ones to their 5,6,7,8-tetrahydro derivatives with large excesses of sodium borohydride[5] made us use this reagent to prepare 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-thione (11). Unlike previous reactions where the 6,7-*cis* isomers were formed exclusively, this product was a 1·6 : 1·0 mixture of *cis* and *trans* isomers. As in previous reductions we were unable, however, to obtain the reduced pteridines completely free from small amounts of boric acid or sodium borate which did not interfere with enzyme activity.[4] The reduced pteridines were free from organic impurities other than solvent which adheres tenaciously. In an attempt to obtain preparations free from borate we reduced the 2-amino-6,7-dimethylpteridin-4(3*H*)-thione by hydrogenation with platinum oxide in methanol. This gave only the *cis*-5,6,7,8-tetrahydro derivative of (11) (by n.m.r.). Similar reduction in trifluoroacetic acid yielded a 2·3 : 1·0 mixture of *cis* and *trans* isomers. The sharp signals in the n.m.r. spectra of these products were an indication that they were free from platinum or platinum complexes.

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one was reduced to the *cis*-hexahydro derivative (12) with excess sodium borohydride. However, when it was reduced catalytically with platinum oxide in methanol, 3M hydrochloric acid or trifluoroacetic acid, hydrogen uptake was not complete and the corresponding 6,7-dimethyl-2-thioxo-1,2,7,8-tetrahydropteridin-4(3*H*)-one only was formed. The 7,8-dihydro structure of this compound was deduced by analogy with the incomplete catalytic reduction products of related pteridines which were known to be the more stable 7,8-dihydro derivatives (compared with the 5,6- or 5,8-dihydro derivatives). The structures of the 7,8-dihydropteridines were deduced by unambiguous synthesis.[8] Catalytic reduction of 6,7-dimethyl-2-methylthiopteridin-4(3*H*)-one with platinum oxide in trifluoroacetic acid also gave the 7,8-dihydro derivative, but when the solvent was methanol or (D_4) methanol (with deuterium gas) reduction was complete and the *cis*-derivatives (13) and (14) were obtained respectively. These were soluble in chloroform which is unusual for oxopteridines.

Preparation of Substrates and Dihydropteridine Reductase Activity

This enzyme has a strict requirement for quinonoid dihydropteridines, e.g. structure (1), as substrates. These substrates are reduced enzymically to the respective 5,6,7,8-tetrahydropteridines at the expense of NADH which in turn is oxidized to NAD^+ (see[1]). The quinonoid species can be generated by specific oxidations (e.g. with peroxidase–hydrogen peroxide or air, potassium ferricyanide, bromine) of the corresponding 5,6,7,8-tetrahydropteridines. Although many of them can be identified by their characteristic u.v. spectra, and by their reactions and n.m.r. spectra at low temperature, they are short lived. They either rearrange to the more stable tautomers, the 7,8-dihydropteridines (24), or undergo degradation depending on the nature and position of the substituents.[1] Neither the tetrahydro nor the 7,8-dihydro derivatives, nor the parent pteridines, are substrates for the enzyme. Consequently,

[8] Armarego, W. L. F., and Schou, H., *J. Chem. Soc., Perkin Trans. 1*, 1977, 2529.

when a tetrahydropteridine is oxidized by the specific oxidants and the transient species formed can be enzymically reduced back to the original tetrahydropteridine at the expense of NADH we can presume that the transient species have a quinonoid structure such as (1).

In order to ascertain that the transient species were substrates we had to show that (i) maximum enzyme activity (i.e. initial rates of oxidation of NADH) was observed at least immediately after addition of enzyme, (ii) at a set concentration of substrate and NADH, enzyme activity increased linearly with increase in enzyme concentration, (iii) at a constant enzyme and NADH concentration but varying substrate concentration, the initial rates against substrate concentrations were hyperbolic, i.e. indicative of saturation kinetics, and (iv) that Lineweaver–Burk plots of the data in (iii), i.e. reciprocal of initial velocity against reciprocal of concentration yielded the kinetic parameters (apparent Michaelis constants K_m and maximum velocities V_{max}). We had shown that the tetrahydropteridin-4(3*H*)-ones (4), (6) and (8) were oxidized by peroxidase–hydrogen peroxide to the transient species (15)–(17) respectively.

Table 1. Kinetic parameters of human brain dihydropteridine reductase in 0·1 M Tris–HCl buffer pH 7·4 at 25°

7,8-Dihydropteridine-4(6*H*)-one	K_m (μM)	V_{max}[A]	V/K	NADH (μM)
2-Amino-6,7-dimethyl, (1)	24·7(±0·6)	199(±2·1)	8·06	95
2,6,7-Trimethyl, (18)	38·8(±0·7)	10·1(±0·1)	0·26	111
6,7-Dimethyl-2-methylthio, (25)	85·5(±0·04)	83·1(±0·3)	0·97	106
6,7-Dimethyl-2-methylamino, (26)	127(±5)	150(±4)	1·18	117
2-Amino-6,8-dimethyl,[B] (29)	349(±26)	24·3(±2·6)	0·06	108

[A] Calculated on μM NADH oxidized per min per mg of protein.

[B] Formed from 6,8-dimethyl-5,6,7,8-tetrahydropterin hydrochloride (Armarego, W. L. F., and Milloy, B. A., *Aust. J. Chem.*, 1977, **30**, 2023), ϵ_{270} 14450 in 4 mM HCl (Pfleiderer, W., and Mengel, R., *Chem. Ber.*, 1971, **104**, 2293), in the assay.

These transient species were substrates for dihydropteridine reductase and we had determined their kinetic parameters.[4] We report an extension of these studies in which we have examined the viability of the derivatives (18), (25), (26) and (29) as substrates for dihydropteridine reductase. The results are summarized in the form of kinetic parameters in Table 1. The parameters of the known substrate 2-amino-6,7-dimethyl-7,8-dihydropteridin-4(6*H*)-one (1), were redetermined under identical conditions and were for comparison. The V/K values have been included in Table 1 because they are a better index of the overall efficiency of the enzyme with a particular substrate than the K_m and V_{max} values (see below). When we oxidized the related *cis*-2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (10) with peroxidase–hydrogen peroxide we found that the quinonoid species (18) was formed at a relatively slow rate which was rate limiting. However, when the oxidant was replaced by three molar, or more, equivalents of potassium ferricyanide (with respect to the pteridine), the oxidation was complete within five seconds. By using the latter oxidant we showed, by the above four criteria, that the transient quinonoid dihydropteridine (18) formed was a substrate (Table 1). Similarly we have shown that the quinonoid pteridines (25) and (26) derived from oxidation of 2-methylthio- (13) and 2-methylamino- (kindly supplied by Dr P. Waring) 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one respectively are also substrates (Table 1). Kaufman[9] had previously demonstrated

that the methylamino compound (26) was a substrate by measuring only *one* initial rate. This rate was 36% of the initial rate of the parent compound (1) measured under identical conditions (compare with Table 1; V/K values are a good index of substrate reactivity in these cases, the larger the value the better the substrate). The two quinonoid species (25) and (26) had typical u.v. spectra (cf.[1]). We had previously shown that the latter rearranged to the 7,8-dihydro tautomer with a half life of 34·6 min (pH 7·6 Tris–HCl buffer, k_{obs} $2{\cdot}0\times10^{-2}$ min^{-1}) and when the rate was compared with that of the 6,7-dideuterated derivative the k_H/k_D value was 9·0.[10] In the present study the 2-methylthio analogue (25) rearranged similarly, but with a half life of 0·9 min (pH 7 Tris–HCl buffer, k_{obs} 0·75 min^{-1}) and when compared with the rate of rearrangement of the deuterated quinonoid dihydropterin derived from (14) the k_H/k_D value was 18. This further confirms the quinonoid structures (25) and (26) because these rearrangements are known to proceed with very large deuterium isotope effects.[1] The 7,8-dihydro compounds formed were slowly further oxidized to the respective pteridines by air.

The thioxopteridinone (12) was oxidized by peroxidase–hydrogen peroxide or potassium ferricyanide as observed by the u.v. spectral changes. However, no dihydropteridine reductase active species could be detected, and the t.l.c. and u.v. spectral properties of the final oxidation product were similar to those of the original 6,7-dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one. This experiment demonstrates that either the quinonoid species (27) were formed in very small (steady state) concentrations or were not formed at all. It should be pointed out that 6-methyl-5,6,7,8-tetrahydro-lumazine [the 2-oxygen analogue of (12)] has been oxidized to 6-methyl-7,8-dihydropteridin-2(3*H*),4(6*H*)-dione [related to the 2-oxygen analogue of (27)] which was not a substrate or inhibitor of the enzyme. We examined the oxidation of the tetrahydrothiopterin (11) with the above two oxidants, and the t.l.c. properties of the products after various intervals of time were similar. The intermediate (28) proved to be a substrate for the enzyme according to the first three criteria above. However, attempts to determine the kinetic parameters were unsatisfactory because it was not possible to reproduce the initial rate traces to within the accepted standard deviation. This is possibly due to the facile oxidation of the 4-thio group to give the respective disulfide which may or may not be enzyme active and/or dismutation between the species (28) and the unchanged 4-thioxo compound (11) in the early stages of oxidation.

The ability of 6,8-dimethyl-7,8-dihydro(6*H*)pterin (29) to act as a substrate for dihydropteridine reductase was also examined in order to find out the effect of methyl substitution at N 8. This compound proved to be a substrate, albeit the poorest of the active pteridines that were studied, but satisfied the criteria for substrate activity stated above.

We conclude that the enzyme can tolerate various substituents in the 2-position, as well as an 8-methyl group, in the quinonoid dihydropteridine substrates with a 4-oxo or 4-thioxo group. The results exclude the possibility that the tautomer with the exocyclic double bond at position 2 [e.g., structure (2)] is a substrate at the active site because such a tautomer cannot be formed when the 2-position is unsubstituted [cf. compounds (15)–(17)], and when it is substituted with a methyl

[9] Kaufman, S., *J. Biol. Chem.*, 1964, **239**, 332.
[10] Armarego, W. L. F., and Waring, P., *J. Chem. Soc., Perkin Trans. 2*, 1982, 1227.

(18) or a methylthio (25) group. The tautomer with a double bond between C 8a–N 8 [e.g., structure (3)] is also excluded on the grounds that its presence cannot be detected in the ^{1}H n.m.r. spectra in neutral solution,[3] kinetic evidence[10] and because the 8-methyl pterin (29) cannot form this tautomer at pH 7·3. The oxidation of 6,8-dimethyl-5,6,7,8-tetrahydropterin to yield (29) should produce a cation with the positive charge on N 8 if it formed a tautomer such as (3); but at neutral pH this should deprotonate at N 3 to form the neutral species (29). The evidence that the pteridine substrates are not in the protonated states on the enzyme had been presented by us previously.[4] The above study also demonstrates that the predominant tautomer in aqueous solution at pH ~ 7 [structure (1), with the endocyclic C 2–N 3 double bond] is the active substrate for dihydropteridine reductase.

Experimental

Microanalyses were determined by the Australian National University Analytical Unit. Analytical specimens were dried at 25° and 0·3 mmHg in the presence of potassium hydroxide unless otherwise stated. ^{1}H and ^{13}C n.m.r. spectra were measured on a Jeol FX 90Q spectrometer operating at 34°. Sodium 3-(trimethylsilyl)propionate was used as internal standard for the ^{1}H n.m.r. spectra (δ values are in ppm and J values in Hz). Kinetic measurements were performed on a Unicam SP1800 double-beam spectrometer and Rikadenki B-281H recorder with 5 mV across the slide wire to produce a maximum pen movement corresponding to 0·1 absorbance units. For higher sensitivity a Cary 219 spectrometer adjusted to produce maximum pen movement of 0·01 absorbance units was used and accurate absorbance for concentration measurements were performed on a Perkin–Elmer Lambda 1 single-beam spectrometer. The cell holders were kept at constant temperature (25°) with recirculating water in a Coolnics thermostat. The mass spectra were measured at 16 eV on a Hitachi M-70 mass spectrometer. Thin-layer chromatography (t.l.c.) was run on Silica gel 60 F_{254} (Merck) and eluted with BuOH/AcOH/H_2O 20 : 3 : 7. The spots were revealed by u.v. light (at 250 and 365 nm) and staining with iodine vapour. I.r. spectra (KBr disc) were measured on a Unicam SP 1050.

Synthesis of Pteridin-4(3H)-ones

5,6-Diaminopyrimidin-4(3*H*)-one, m.p. 238–243° (dec.), was prepared by desulfurizing the respective 2-thio derivative[11] and was recrystallized from water (Found: C, 33·9; H, 5·6; N, 39·8. $C_4H_6N_4O.0·8H_2O$ requires C, 34·2; H, 5·5; N, 39·9%). The ^{1}H n.m.r. spectrum had δ (D_2O): 7·76; and ϵ_{278} 8912 (pH 7). Pteridin-4(3*H*)-one, prepared from the above pyrimidinone and glyoxalbis(sodium bisulfite),[11] was purified by alumina (Brockman II, B.D.H.) column chromatography and eluted with 18 mM aqueous ammonia. The main fraction (monitored by t.l.c.) was evaporated, the residue dissolved in hot water (charcoal), the pH was adjusted to 3–4 and cooled. The solid was collected, crystallized from a large volume of acetic acid and sublimed at 320°/0·9 mmHg (Found: C, 49·0; H, 2·7; N, 38·1. $C_6H_4N_4O$ requires C, 48·7; H, 2·7; N, 37·8%). ^{1}H n.m.r. (1 M DCl): 8·97, d, J 2·3, H 6 or 7; 9·05, d, J 2·3, H 7 or 6; 9·10, s, H 2; (1 M NaOD): 8·46, s, H 2; 8·74, d, J 2·2, H 6 or 7; 8·85, d, J 2·2, H 7 or 6. (*6,7-D_2*)-*Pteridin-4(3H)-one*, m.p. 303–355° (dec.), was similarly prepared by using (1,2-D_2)-glyoxalbis(sodium bisulfite)[12] and sublimed at 230°/0·7 mmHg (Found: C, 48·2; H + D, 4·2; N, 37·5; *m/e* 149. $C_6H_2D_2N_4O$ requires C, 48·0; H + D, 4·2; N, 37·3%; M^+ 150). ν_{max} 3090 (3030), 2925 (2885), 1724 (1724), 1615 (1617), 1545 (1550), 1434 (1468), 1378 (1398), 1322 (1320), 1268 (1280), 1210 (1228), 1182 (1163), 1150 (1108) 1013 (1032), 1000, 990, 925 (923), 836 (827), 710 (720) and 652 (607) cm^{-1}, the values for the unlabelled ^{1}H-containing compound above being the ones in parentheses. The ^{1}H n.m.r. spectrum indicated *c.* 3·7% contamination with the ^{1}H-containing compound. 5,6,7,8-Tetrahydropteridin-4(3*H*)-one[5] (4), prepared by reduction of pteridin-4(3*H*)-one with $NaBH_4$, always contained small amounts of

[11] Albert, A., Brown, D. J., and Cheeseman, G., *J. Chem. Soc.*, 1951, 474.
[12] Armarego, W. L. F., Randles, D., and Taguchi, H., *Biochem. J.*, 1983, **211**, 357.

borate (green-edged flame test with H_2SO_4/EtOH) although it gave one spot on t.l.c. (Found: C, 41·5; H, 5·7; N, 31·8. $C_6H_8N_4O.0·1NaH_2BO_3.H_2O$ requires C, 41·2; H, 5·6; N, 32·0%). The formula weight is consistent with the reported ϵ_{289} value of 8511 (pH 7·0) for the anhydrous neutral species; ^{1}H n.m.r. (1 M DCl): 3·69, m, H 6,6,7,7 and 8·59, s, H 2. Similar reduction of (6,7-D_2)-pteridin-4(3*H*)-one with $NaBD_4$ in D_2O gave (6,6,7,7-D_4)-pteridin-4(3*H*)-one (5) (Found: C,41·4; H + D, 8·55; N, 31·5; *m/e* 156, base peak. $C_6H_4D_4N_4O.0·02NaH_2BO_3.H_2O$ requires C, 41·0; H + D, 8·1; N, 31·9%; M^+, 156]. The u.v. spectra and t.l.c. properties were identical with the above. The ^{1}H n.m.r. spectrum indicated that it was better than 95% deuterated on C 6 and C 7.

6-Methylpteridin-4(3*H*)-one[13] (150 mg) in methanol (300 ml) and platinum oxide (250 mg pre-reduced in 10 ml of methanol) were shaken with hydrogen (25°/720 mmHg) for 4·2 h, a further quantity of platinum oxide (100 mg pre-reduced in 10 ml of methanol) was added and shaking was continued for a further 1·5 h. The solution was filtered and evaporated to dryness. The residue (144 mg) was recrystallized from water (10 parts) to give 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (6), m.p. >211° (dec.) (Found: C, 45·9; H, 6·3; N, 29·9. $C_7H_{10}N_4O.H_2O$ requires C, 45·65; H, 6·6; N, 30·4%) which was pure by t.l.c., and had u.v. spectra as reported.[5] Reduction with $NaBH_4$ (instead of KBH_4 as reported)[5] also gave the 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one which could not be freed for small amounts of borate (flame test) (Found: C, 36·4; H, 5·4; N, 24·0. $C_7H_{10}N_4O.0·76NaH_2BO_3.0·1H_2O$ requires C, 36·3; H, 5·1; N, 24·2%). The ^{1}H n.m.r. spectrum [1 M DCl): 1·48, d, *J* 6·3, 6-Me; 3·39, q, J_{gem} −14·4, J_{vic} 9·4, H 7$_{ax}$; ~ 3·7, m, H 6; 3·77, q, J_{gem} −14·5, J_{vic} 3·0, H 7$_{eq}$; 8·2, s, H 2] indicates a conformation similar to that of 6-methyl-5,6,7,8-tetrahydropterin, i.e. the methyl group is predominantly equatorial.[8] When 6-methylpteridin-4(3*H*)-one (200 mg) in trifluoroacetic acid (5 ml) and platinum oxide (50 mg pre-reduced in 2·5 ml of trifluoroacetic acid) was shaken with hydrogen (25°/720 mmHg), the reduction was very rapid, and after 2 h the catalyst was filtered off, the filtrate was added to a cold solution of methanol (1·8 ml) and 7 M methanolic HCl (0·4 ml) followed by dry ether (60 ml) whereby the colourless hydrochloride separated. It was collected and washed with ether (3×15 ml) by centrifugation and dried *under vacuum*. *6-Methyl-2,3,5,6,7,8-hexahydropteridin-4(1H)-one* (19) *hydrochloride* (242 mg) was recrystallized from methanol containing a few drops of methanolic HCl and ether, and had m.p. 213–218° (effervescence) (Found: C, 35·5; H, 6·1; Cl, 21·6; N, 23·1. $C_7H_{12}N_4O.1·45HCl.H_2O$ requires C, 35·2; H, 6·5; Cl, 21·5; N, 23·4%). ϵ_{258} (inflex) 158 (pH 0); ϵ_{293} 1054 (pH 7·3) and ϵ_{286} 1776 (pH 13·2). ^{1}H n.m.r. (1 M DCl): 1·62, d, *J* 6·2, 6-Me; 3·61, q, J_{gem} −14·2, J_{vic} 10·6, H 7$_{ax}$; 3·95, q, J_{gem} −14·2, J_{vic} 4·4, H 7$_{eq}$; ~4·0, m, H 6; 4·99, s, H 2. ^{13}C n.m.r. (proton coupled) (D_2O, dioxan internal standard 67·6 ppm downfield from Me_4Si): 16·0, q, *J* 131, 6-Me; 44·7, m, C 7; 49·2, m, C 6; 52·1. t, *J* 159, C 2; 157·6, s, C 8a; 162·9, s, C 4. *6-Methyl-(6,7-D_2)-5,6,7,8-tetrahydropteridin-4(3H)-one* (7) was prepared as above but by using $NaBD_4$ in D_2O and contained borate (flame test), m.p. 213–215° (dec.) [Found: C, 44·5; H + D, 7·3; N, 29·3; *m/e* 168 (100%), 153 (75%), 139·3 (metastable). $C_7H_8D_2N_4O.0·1NaH_2BO_3.0·9H_2O$ requires C, 44·0; H + D, 7·3; N, 29·5%; M^+ 168, M^+ − 15 (Me) 153]. The u.v. spectra and t.l.c. properties were identical with the above and the ^{1}H n.m.r. spectrum indicated better than 95% deuteration. The concentration of aqueous solutions were determined from ϵ_{290} 9685 (pH 7·0).

6,7-Dimethylpteridin-4(3*H*)-one[13] [500 mg, ^{1}H n.m.r. (1 M DCl): 2·78, s, 7-Me or 6-Me; 2·81, s, 6-Me or 7-Me; 9·23, s, H 2] in 3M HCl (25 ml) and platinum oxide (100 mg pre-reduced in 25 ml of 3 M HCl) were shaken with hydrogen as above (5·25 h). The catalyst was filtered off and the filtrate evaporated to dryness. The colourless residue was dissolved in methanol (23 ml) and methanolic HCl (7 M, 18 ml) was added. The cis-*6,7-Dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one* (8) *hydrochloride* (500 mg) that separated on cooling, was collected and dried under vacuum, m.p. >219° (dec.) (Found: C, 36·7; H, 6·1; Cl, 25·5; N, 21·3. $C_8H_{12}N_4O.1·87HCl.0·7H_2O$ requires C, 36·7; H, 6·1; Cl, 25·5; N, 21·3%). U.v. spectrum had ϵ_{262} 6680 (pH 0·0) and ϵ_{292} 9025 (pH 7·0). ^{1}H n.m.r. (0·5 M DCl): 1·28, d, *J* 6·6, 6-Me or 7-Me; 1·35, d, *J* 6·6, 7-Me or 6-Me; 3·85 and 3·93, m, *J* 6·6 and 3·3, H 6 and 7; 8·09, s, H 2. *6,7-Dimethyl-(6,7-D_2)-5,6,7,8-tetrahydropteridin-4(3H)-one* (9) *hydrochloride*, m.p. >218° (dec.) was similarly obtained by using 3 M DCl in D_2O and D_2 gas [Found: C, 34·3; H + D, 6·6; Cl, 25·2; N, 19·7; *m/e* 182

[13] Albert, A., Brown, D. J., and Cheeseman, G., *J. Chem. Soc.*, 1952, 4219.

(87%), 167 (100), 152 (75), 138 (55). $C_8H_{10}D_2N_4O.2\,HCl.1{\cdot}4H_2O$ requires C, 34·3; H + D, 6·7; Cl, 25·3; N, 20·0%; M^+ 182, $M^+ - 15$ (Me) 167, $M^+ - 30$ (2Me) 152, $M^+ - 44$ 138]. Its ^1H n.m.r. spectrum indicated better than 95% deuterium incorporation at C 6 and C 7.

5,6-Diamino-2-methylpyrimidin-4(3*H*)-one sulfate[14] (0·5 g) and diacetyl (0·5 ml) in water (10 ml) was boiled under reflux for 1·5 h, the pH was adjusted to 4 and cooled. The *2,6,7-trimethylpteridin-4(3H)-one* (138 mg, 55%) that separated was washed with ethanol, dried and had m.p. >241° (dec.) (Found: C, 54·5; H, 5·3; N, 28·5. $C_9H_{10}N_4O$ requires C, 54·8; H, 5·5; N, 28·4%). ^1H n.m.r. (D_2O): 2·54, s, 2-Me; 2·66, s, 6-Me or 7-Me; 2·68, s, 7-Me or 6-Me. Platinum oxide (50 mg) in 3 M hydrochloric acid (5 ml) was pre-reduced by shaking with hydrogen at 20°/720 mmHg, the trimethylpteridinone (50 mg) was added, and hydrogenation was continued until completion (9·5 h). Filtration and evaporation of the filtrate to dryness gave a solid (70 mg) which was dissolved in 7 M methanolic hydrogen chloride and a small volume of ether was added to cause the separation of cis-*2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one* (10) *hydrochloride* (30 mg), m.p. 210·5–211·5° (dec.) (Found: C, 37·2; H, 6·3; Cl, 23·7; N, 19·1. $C_9H_{14}N_4O.2\,HCl.1{\cdot}4H_2O$ requires C, 37·1; H, 6·5; Cl, 23·9; N, 19·2%). ϵ_{263} 8280 (4 mM HCl). ^1H n.m.r. (D_2O): 1·26, d, *J* 6·6, 6-Me or 7-Me; 1·31, d, *J* 6·6, 7-Me or 6-Me; 2·34, s, 2-Me; 3·81, m, *J* 6·6 and 3·3, H 6 and 7.

cis-6,7-Dimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3*H*)-one hydrochloride supplied by Dr P. Waring had ϵ_{270} 13 800 (4 mM HCl).

Syntheses of Thiotetrahydropteridines

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one[15,16] [0·5 g, ^1H n.m.r. (2 M DCl): 2·64, s, 6-Me or 7-Me; 2·68; s, 7-Me or 6-Me (1 M NaOD): 2·47, s, 6-Me and 7-Me] in 1 M sodium carbonate (5 ml) was reduced by boiling under reflux with sodium borohydride (6 g, added in small portions) until the u.v. spectrum in 1·5 M hydrochloric acid indicated complete reduction (15 h), and cooled. The solid (0·26 g) was collected, washed with water (centrifuge) and dried. The solid (0·15 g) was dissolved in methanol (15 ml), ethanol (15 ml) was added, the solution was filtered and cooled. The yellow solid was collected, washed with small volumes of methanol and dried to give colourless cis-*6,7-dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one* (12) (28 mg), m.p. >259° (dec.) (Found: C, 41·7; H, 5·6; N, 23·3; S, 13·2. $C_8H_{12}N_4OS.0{\cdot}5CH_3OH.0{\cdot}1H_2O.0{\cdot}18NaH_2BO_3$ requires C, 41·6; H, 6·0; N, 22·8; S, 13·1%). ^1H n.m.r. (0·01 M NaOD): 1·07, d, *J* 6·7, 6-Me or 7-Me; 1·08, d, *J* 6·7, 7-Me or 6-Me; 3·24, octet, *J* 6·7 and 3·2, H 6 or 7; 3·55, octet, *J* 6·7 and 3·2, H 7 or 6. ϵ_{282} 5342 (pH 0). Reduction with platinum oxide in 3 M hydrochloric acid, methanol or trifluoroacetic acid (5–11 h) gave the 6,7-dimethyl-2-thioxo-3,4,7,8-tetrahydropteridin-4(1*H*)-one [^1H n.m.r. (5 M DCl): 1·55, d, *J* 7·1, 7-Me; 2·53, s, 6-Me; 4·94, q, *J* 7·1, H 7] which was oxidized by air in the solid state and in solution at room termperature to give the parent pteridinone.

6,7-Dimethyl-2-methylthiopteridin-4(3*H*)-one, m.p. >255° (dec.) was prepared in 63% yield by boiling the preceding thioxo compound (5 g) in 0·5 M sodium hydroxide (50 ml) with methyl iodide (4·42 ml) for 15 min, cooling, filtering and recrystallizing the product from propan-1-ol [lit.[15,17] m.p. 283° (dec.) prepared by condensation of 5,6-diamino-2-methylthiopyrimidin-4(3*H*)-one and diacetyl] (Found: C, 49·0; H, 4·6; N, 25·4; S, 14·1. Calc. for $C_9H_{10}N_4OS$: C, 48·6; H, 4·5; N, 25·2; S, 14·4%). ^1H n.m.r. (0·1 M NaOD): 2·54, s, MeS; 2·60, s(br), 6-Me and 7-Me and (2 M DCl): 2·70; s, 6-Me and 7-Me; 2·83, s, MeS. Attempts to prepare the tetrahydro derivative as above required very large amounts of sodium borohydride and gave a very impure product that released methanethiol readily on attempted purification. Smaller amounts of borohydride were required when propan-1-ol was used but purification was difficult. cis-*6,7-Dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one* (13) (67 mg), m.p. >115° (dec.), was obtained when the methylthiopteridinone (50 mg) in methanol (5 ml) was hydrogenated with pre-reduced platinum oxide (45 mg in 5 ml methanol) at 20°/720 mmHg (7·5 h). The catalyst was filtered off and the filtrate was evaporated (Found: N, 17·0; S, 9·5. $C_9H_{14}N_4OS.5{\cdot}6H_2O$ requires N, 17·1, S, 9·8%). ^1H n.m.r. ($CDCl_3$): 1·18, d, *J* 6·6, 6-Me or 7-Me; 1·22, d, *J* 6·6, 7-Me or 6-Me;

[14] Albert, A., Brown, D. J., Wood, H. C. S., *J. Chem. Soc.*, 1954, 3832.
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[16] Gal, E. M., *J. Am. Chem. Soc.*, 1950, **72**, 3532.
[17] Angier, R. B., and Curran, W. V., *J. Am. Chem. Soc.*, 1959, **81**, 5650.

2·49, s, MeS; 3·49, octet, *J* 6·6 and 3·0, H 6 or 7; 3·74, octet, *J* 6·6 and 3·0, H 7 or 6. ϵ_{274} 6710 (1·5 M HCl). Similar reduction in CD_3OD and D_2 gas gave the *6,7-dideutero derivative* (14) which had >96% deuterium incorporation (by n.m.r.). Reduction with platinum oxide in trifluoroacetic acid gave *6,7-dimethyl-2-methylthio-7,8-dihydropteridin-4(3H)-one* which was isolated as the hydrochloride: ^{1}H n.m.r. (5 M DCl): 1·57, d, *J* 7·1, 7-Me; 2·58, s, MeS; 2·95, s, 6-Me; 4·99, q, *J* 7·1, H 7.

2,5,6-Triaminopyrimidin-4(3*H*)-thione, prepared by direct thiation of the respective pyrimidin-4(3*H*)-one sulfate as before,[18] was very impure and was purified by placing it (2 g, in 150 ml water) on a Dowex 50W×4 (400 ml, 20–50 mesh, H^+ form) column, washing it with water (1·5 l.), 0·5 M hydrochloric acid (0·5 l.) and 1·5 M hydrochloric acid (8 l.), and was eluted with 2 M hydrochloric acid (10 l., followed by the u.v. spectral band at 310 nm). Evaporation gave a residue which was triturated with the latter solvent (*c.* 4 ml), filtered off and dried at 100° for 2 h to give the colourless *thione hydrochloride* (1·32 g), m.p. >300° (dec.) (Found: C, 21·1; H, 3·9; Cl, 30·6; N, 30·6; S, 13·8. $C_4H_3N_5S.2HCl$ requires C, 20·9; H, 3·9; Cl, 30·8%; N, 30·4; S, 13·9; lit.[18] reported the free base). This hydrochloride (200 mg) was dissolved in water (5 ml), the pH was adjusted to 4 and diacetyl (0·2 ml) was added and boiled under reflux (70 min). The solid (150 mg) that separated on cooling was collected and recrystallized from water to give *2-amino-6,7-dimethylpteridin-4(3H)-thione* (140 mg), m.p. >290° (dec.) (Found: C, 45·9; H, 4·4; N, 33·3; S, 14·5. $C_7H_9N_5S.0·15H_2O$ requires C, 45·8; H, 4·5; N, 33·4; S, 15·3%). ^{1}H n.m.r. (1 M DCl): 2·66, s, 6-Me or 7-Me; 2·68, s, 7-Me or 6-Me. The thione (50 mg) was reduced with sodium borohydride in sodium carbonate as above except that at the end of the reaction (12 h, under nitrogen) 7 M methanolic hydrogen chloride was added (ice bath) and evaporated to dryness. The residue (2·33 g) was passed through a Dowex 50W×8 (2 ml, 100–200 mesh) column, washed with 0·5 M to 1·5 M hydrochloric acid, and eluted with 2 M hydrochloric acid. Evaporation of the last eluate gave a mixture of cis- and trans-*2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione* (11) *hydrochloride* (1·6 : 1·0 by n.m.r.) (60 mg), m.p. >211° (dec.) (Found: C, 26·3; H, 4·7; Cl, 23·4; S, 9·5. $C_8H_{13}N_5S.1·6HCl.0·8\ NaCl.0·8\ H_3BO_3$ requires C, 26·3; H, 4·7; Cl, 23·3; S, 8·8%); ^{1}H n.m.r. (2 M DCl at 270 MHz): *cis* isomer 1·33, d, *J* 6·8, 6-Me or 7-Me; 1·41, d, *J* 6·8, 7-Me or 6-Me; 3·98, m, *J* 6·8 and 3·3, H 6 or 7; 4·05, m, *J* 6·8 and 3·3, H 7 or 6; *trans* isomer 1·42, d, *J* 6·6, 6-Me or 7-Me; 1·53, d, *J* 6·6, 7-Me or 6-Me; 3·56, m, *J* 6·6 and 8·3, H 6 or 7; 3·74, m, *J* 6·6 and 8·3, H 7 or 6, assigned by proton decoupling. Catalytic reduction with platinum oxide in trifluoroacetic acid (11 h) also gave the *cis* and *trans* mixture (2·3 : 1·0 by n.m.r.) of the tetrahydro-pteridinthione hydrochloride, whereas catalytic reduction in methanol gave exclusively the *cis* isomer (by n.m.r.).

Dihydropteridine Reductase Assay

The assays were performed in cuvettes in the thermostated cell holders of a double-beam spectrometer set at 340 nm. Stock solutions containing 1 M Tris–HCl (100 μl), 2 mM potassium ferricyanide (100 μl) and water (670 μl) were placed in each cuvette. The substrate in 4 mM hydrochloric acid (100 μl, final concentration 0·1 to ~ 1·5K_m) was added to each cuvette and allowed to equilibrate (*c.* 1 min). NADH (30 μl, with final concentrations listed in Table 1) was added simultaneously to the cuvettes by using two platinum buckets which were then moved up and down (10 times) into the assay solution to ensure complete mixing. One of the buckets also contained human brain dihydropteridine reductase[19] (3–8 μl, 0·118–0·314 μg). Soon after mixing the pen recorder was activated. The initial rates of the reaction from at least five different concentrations of pteridine substrates were calculated from the rate traces and using ϵ_{340} 6200 for the extinction coefficient of NADH. The K_m and V_{max} values (see Table 1) were computed from the initial rates and concentrations of substrates by means of the Cornish–Bowden program.[20] In preliminary runs we showed that at the highest concentrations of potassium ferricyanide and NADH the rate of oxidation of the latter was neligible compared with the enzymic rate. Also the largest non-enzymic rate of oxidation of NADH by the highest concentration of quinonoid dihydropteridine was small (<5%) compared with the enzymic rate.

[18] McCormack, J. J., and Mautner, H. G., *J. Org. Chem.*, 1964, **29**, 3370.
[19] Armarego, W. L. F., and Waring, P., *Biochem. Biophys. Res. Commun.*, 1983, **113**, 895.
[20] Cornish-Bowden, A., and Endrenyi, L., *Biochem. J.*, 1981, **193**, 1005.

and both these non-enzymic rates are compensated in the double beam of the spectrometer, i.e. the loss in NADH concentration was small and the concentration of NADH was still at or near saturating levels.

The rate of rearrangement of quinonoid dihydropteridines to the 7,8-dihydropteridines were determined by generating the quinonoid species from the respective 5,6,7,8-tetrahydropteridines with ferricyanide as above and observing the rate of change of absorbance at 340 nm, a wavelength at which the difference in ϵ values between $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ is very small compared with the total change in absorbance during the rearrangement.

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